Annals of Biochemistry and Experimental Medicine

Volume I, 1941.

Edited by

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A STUDY OF THE NUTRITIONAL VALUE OF THE COOKED DIETS AS CONSUMED BY STUDENTS IN CALCUTTA WITH REFERENCE TO SEASONAL VARIATIONS

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In most parts of the world the nutritional level has been partly judged by a survey of the raw food-stuffs that are purchased and cooked. Wilson *et al* (1) made a diet survey of some families and institutions in Calcutta by this method. Diet surveys in some South Indian villages have also been carried out on similar lines by Aykroyd *et al* (2). This method of survey, however, is far from satisfactory. The tables from which the values are calculated are based on analyses of raw food-stuffs. The soil and climate have got considerable influence over the nutritive value of food-stuffs. The analyses of raw food-stuffs obtained from different parts of the country may and do vary considerably and so the values calculated may not be accurate. Again, the whole quantity of food purchased is not consumed. Portions which are not edible are rejected. Therefore if the nutritive values are evaluated on the weight of the food-stuffs purchased, deducting a certain arbitrary fraction as waste, which is the usual practice in the survey method, it may not give a true picture of the amounts of the dietary ingredients consumed. Further, cooking is a fairly complicated process particularly in Bengal households and is likely to materially alter the nutritional value of the diet both quantitatively and qualitatively. For instance, the author (3) has shown that there is a considerable loss of minerals when the rice is cooked and the rice water is thrown away, which is the usual practice in this country. For the above reasons we have considered it desirable to analyse the cooked diets as they are actually consumed. A study of cooked diets obtained from certain middle class students' hostels, families and hospitals in Calcutta was undertaken by Pal and Guha (4). The study was not complete because the diets in different seasons could not be considered and the available iron and the available phosphorus values were not determined. According to the present state of knowledge only the ionisable iron can be assimilated and is therefore available. The diet may contain enough total iron but if it does not contain sufficient ionisable iron most of the total iron would be unavailable. Therefore the ionisable iron determination is essential in order to know the actual iron consumption. Of the total phosphorus also, that part which is present as phytin is either wholly or largely unavailable from the nutritional point of view. It is therefore

preferable to treat the difference between the total phosphorus and phytin phosphorus as the available phosphorus consumption. Hence both the total and phytin phosphorus should be determined. We considered that an investigation of the cooked diet actually consumed carried out in three distinct seasons and in each season for a period of at least seven consecutive days was necessary in order to obtain a more representative picture of the diets that are consumed in the hostels throughout the year.

In the present investigation the cooked diets from thirteen different hostels in Calcutta were analysed in three different months namely, April (1939), August (1938) and December (1939). These months were selected because they are equally spaced and represent three distinct seasons, namely, the spring, the monsoon and the winter of the year. During each season we have analysed the fore-noon and the evening meals—the two principal meals —consumed by an average student for a period of seven consecutive days. The refreshments of the students which add an uncertain value to the students' dietaries were not considered in these investigations. The individual variations in the consumption of food from student to student in the same hostel were not considered in the present study due to lack of resources at our disposal.

METHOD OF SAMPLING

. Fore-noon and evening meals from each hostel were taken for a period of seven consecutive days. Separate items of each diet per head per meal were taken as follows. 450 g. of cooked rice and 125 g. of aqueous decoction of pulses (dal) were taken in every season as the average consumption per head per meal except in a girls' hostel where average consumption was found to be 300 g. rice and 100 g. of aqueous decoction of pulses. Vegetable and animal curries were found to be consumed in varying quantities in different hostels and in each hostel on different days. The ranges of variation of the daily consumption of animal food (fish, meat, eggs but not milk or milk-products) from hostel to hostel were found to be 74−164 g. in April, 58−126 g. in August and 69−213 g. in December. The ranges of variation of the daily consumption of vegetables for the corresponding months were 113−406 g., 119−311 g. and 86−360 g. respectively. On an average the consumption of vegetable and animal curries during the month of August thus tended to be less as compared with the consumption in the other two months. The average consumption of these items of food per head per meal was calculated roughly from the amount of the meal supplied by the super-intendents of the hostels.

Different items of food after weighing were intimately mixed and minced in a stainless mincer. A definite quantity of the well minced food was taken in a porcelain dish and dried in a gas oven at 45°—50°C. The dried mass was then powdered in a porcelain mortar. The weight of the dried food was recorded and it was then sampled in a glass jar.

METHODS

Estimation of protein. Nitrogen was estimated by the usual Kjeldahl's method and the value of protein obtained by multiplying the figure by 6.25.

Estimation of ionisable iron. Ionisable iron was estimated on the undried minced food by Hill's dipyridyl method (5—7).

For the estimation of calcium, total iron, total phosphorus and copper the dried diet mixture was incinerated on a silica crucible. The ash was extracted according to the method of McCance *et al* (8) with dilute hydrochloric acid and the acid extract was made up to a definite volume. Aliquots of this solution were used for the determination of the above minerals.

Estimation of total iron. Iron determination was carried out by the method of Elvehjem (9) as described by Pal and Guha (4).

Estimation of calcium Calcium was estimated by the method of Pal and Guha (4) with the modification that the precipitated calcium oxalate was dissolved in sulphuric acid and titrated with a solution of $N/100$ permanganate.

Estimation of total phosphorus. Total phosphorus was determined by the improved colorimetric method of Berenblum and Chain (10). 1 C.c. of the ash extract was taken in a separating funnel and the following added: 0.5 c.c. of $10N$ H_2SO_4, 2 c.c. of distilled water, 2.5 c.c. of 5% ammonium molybdate and 10 c.c. of *iso*butyl alcohol. The mixture was shaken for 1 to 2 minutes and the aqueous layer was discarded. The alcoholic solution was washed by shaking with N sulphuric acid and shaken with about 15 c.c. of 0.2% stannous chloride solution. The aqueous layer was discarded. The blue solution was poured into a 10 c.c. measuring flask, the separating funnel was washed with ethyl alcohol and the solution made upto the mark with the washings. The blue colour was compared in a colorimeter with the nearest standards, prepared from KH_2PO_4 similarly treated.

Estimation of phytin phosphorus. Phytin was determined on a hydrochloric acid extract of the dried material by the method of McCance *et al* (8). The phytin was precipitated as the insoluble iron salt, digested with sulphuric acid and perchloric acid mixture and the phosphorus in the wet ash was determined by the modified colorimetric method of Berenblum and Chain (10) described above.

Estimation of copper. The metal was determined by a colorimetric method using sodium diethyl-dithiocarbamate as described by McCance *et al* (8).

The mean daily consumption of the food constituents per individual in the different hostels in different seasons is given in Table I.

TABLE I

Daily consumption per individual in the following hostels during the months of April (1939), August (1938) and December (1939) compared.

Hostel No.		Protein (g.)	Ether extract (g.)	Calcium (g.)	Total phosphorus (g.)	Phytin phosphorus (g.)	Total iron (mg.)	Ionisable iron (mg.)	Copper (mg.)
1.	Apr.	65.16	56.23	1.44	1.28	0.45	20.9	6.5	3.6
	Aug.	53.84	39.70	0.54	0.79	0.38	14.4	—	3.8
	Dec.	65.24	53.45	0.76	1.09	0.21	29.3	13.4	1.1
	Average	61.41	49.79	0.91	1.05	0.35	21.5	9.9	2.8
2.	Apr.	66.37	45.33	1.44	1.06	0.41	25.1	5.5	5.7
	Aug.	52.29	38.30	0.64	0.98	0.39	16.3	—	3.0
	Dec.	83.42	51.61	0.94	1.07	0.23	28.7	12.4	1.4
	Average	67.36	45.08	1.00	1.04	0.34	23.3	8.9	3.4
3.	Apr.	63.68	43.10	0.94	1.50	0.38	35.4	5.6	7.6
	Aug.	51.83	32.80	0.63	0.77	0.33	15.4	—	3.4
	Dec.	72.20	53.87	0.98	0.89	0.20	62.6	8.0	2.9
	Average	62.57	43.25	0.85	1.05	0.30	37.8	7.3	4.6
4.	Apr.	56.59	33.29	0.71	0.83	0.32	17.8	9.2	5.8
	Aug.	55.81	18.00	0.66	0.98	0.33	11.4	—	2.5
	Dec.	71.61	59.50	0.53	1.01	0.22	34.3	6.9	1.7
	Average	61.34	36.93	0.63	0.94	0.29	21.2	8.0	3.3
5.	Apr.	58.02	35.62	0.73	0.83	0.32	22.6	8.9	9.7
	Aug.	48.42	20.30	0.45	0.58	0.34	11.4	—	1.9
	Dec.	80.41	60.17	0.73	1.19	0.22	36.8	7.8	1.8
	Average	62.28	38.69	0.63	0.87	0.29	23.6	8.3	6.7
6.	Apr.	66.22	43.25	1.37	0.98	0.50	18.3	5.9	4.2
	Aug.	50.96	29.20	0.62	0.72	0.35	14.0	—	2.8
	Dec.	65.72	42.06	0.52	1.02	0.20	45.0	8.1	1.5
	Average	60.96	38.17	0.84	0.90	0.35	25.8	7.0	2.8

TABLE I (contd.)

Hostel No.		Protein (g.)	Ether extract (g.)	Calcium (g.)	Total phosphorus (g.)	Phytin phosphorus (g.)	Total iron (mg.)	Ionisable iron (mg.)	Copper (mg.)
7.	Apr.	55.75	24.65	0.74	0.93	0.43	25.3	6.6	8.1
	Aug.	44.61	22.90	0.77	0.87	0.30	14.4	—	1.7
	Dec.	64.62	49.83	0.66	0.90	0.20	27.6	10.1	1.8
	Average	54.99	32.46	0.72	0.90	0.31	22.4	8.4	3.7
8.	Apr.	49.20	43.31	1.34	0.76	0.35	23.8	4.3	3.5
	Aug.	41.00	21.50	0.41	0.80	0.31	10.4	—	2.5
	Dec.	63.32	50.15	0.64	0.88	0.21	36.5	12.3	2.0
	Average	51.17	38.32	0.79	0.81	0.29	23.6	7.3	2.7
9.	Apr.	60.95	41.50	0.99	0.96	0.44	21.6	5.7	6.7
	Aug.	45.17	24.30	0.58	0.91	0.32	13.6	—	3.4
	Dec.	48.98	43.25	0.60	0.86	0.22	34.0	7.3	3.2
	Average	51.70	36.35	0.72	0.91	0.33	23.1	6.5	6.6
10.	Apr.	55.59	40.19	1.26	0.84	0.40	25.7	4.5	6.2
	Aug.	41.32	23.20	0.46	0.63	0.34	12.4	—	2.4
	Dec.	58.98	42.11	0.55	0.95	0.19	40.1	8.0	1.3
	Average	51.96	35.17	0.76	0.81	0.31	26.1	6.2	3.3
11.	Apr.	60.11	41.34	0.74	0.78	0.35	13.7	4.7	3.1
	Aug.	47.83	15.20	0.38	0.42	0.26	10.4	—	2.2
	Dec.	53.64	31.25	0.66	0.90	0.18	25.4	8.8	2.9
	Average	53.36	29.26	0.59	0.70	0.26	16.5	6.7	2.7
12.	Apr.	53.58	27.90	1.05	0.78	0.38	18.0	3.6	2.1
	Aug.	51.72	29.30	0.55	0.74	0.31	15.7	—	1.9
	Dec.	58.13	40.60	0.44	0.74	0.20	33.7	5.2	1.2
	Average	54.48	32.60	0.68	0.75	0.30	22.5	4.4	1.7
13.	Apr.	40.93	39.70	1.02	0.73	0.30	18.1	3.8	2.2
	Aug.	29.17	23.70	0.30	0.52	0.23	7.0	—	2.1
	Dec.	31.91	28.19	0.37	0.51	0.10	13.5	5.2	1.3
	Average	34.00	30.53	0.56	0.59	0.21	12.6	4.5	1.9

A summary of the Statistical Report kindly prepared by Prof. P. C. Mahalanobis (which will be published in 'Sankhya", *The Indian Journal of Statistics*) is given below: —

1. The months selected belonged to different years, namely, August in 1938, and April and December in 1939. The statistical analysis was carried out on the assumption that there was no appreciable annual variation for the same month (or season) and that the variations in the total quantities of food in different months were negligible. In the absence of replication, the interaction between hostels and months was used in the analysis of variance in the place of properly estimated residual errors.

2. There were significant differences in the weight of practically all the food constituents studied here in the three months December, 1939, April, 1939 and August 1938.

3. On the whole, December, 1939, had the highest content of all the food constituents except calcium ; April, 1939, had the highest calcium content but came next to December in all the other constituents. August, 1938, apparently had the lowest weights of all the food constituents.

4. There were appreciable differences between hostels on the basis of which it is possible to classify the hostels in broad groups ;

    (*i*)   A group of three hostels is distinctly better than the other hostels.

    (*ii*) Then comes a second group of six or seven hostels.

    (*iii*) Two Moslem hostels occupy the two lowest places among men's hostels.

    (*iv*) A girls' hostel comes last with an appreciably lower content of all the food constituents.

5. Compared to requirements which may be taken as standard, it is found that in December, 1939, there was only a small shortage of protein, ether extract, and calcium with a serious shortage in available phosphorus and ionisable iron. In April, 1939, the calcium content was above the standard requirements ; protein and ether extract are in moderate defect, while available phosphorus and total iron are in large defect. In August, 1938, all the food constituents fell far short of standard requirements.

6. Considering the average of three months we find that calcium consumption was 85 per cent, protein consumption 75 per cent, consumption of ether extract 75 per cent, consumption of available phosphorus 43 per cent and that of ionisable iron 48 per cent of the standard requirements. As regards food constituents studied here the greatest need for improvement thus appears to be in ionisable iron and available phosphorus and then in protein, ether extract and calcium.

TABLE II

Seasonal average for all the hostels and comparison with standard requirements.

Food constituents	Seasonal averages for all hostels				Standard requirement	Seasonal averages in the form of percentages of the standard requirement			
	December 1939	April 1939	August 1938	Average		December 1939	April 1939	August 1938	Average
Protein (g.)	62.94	57.86	47.23	56.00	75.00	84	77	63	75
Ether extract (g.)	46.62	39.65	26.03	37.43	50.00	93	79	52	75
Calcium (g.)	0.64	1.06	0.54	0.75	0.88	73	120	61	85
Phosphorus (g.)	0.92	0.94	0.75	0.87	—	—	—	—	—
Phytin phosphorus (g.)	0.20	0.39	0.32	0.30	—	—	—	—	—
Total phosphorus— Phytin phosphorus (g.)	0.72	0.55	0.43	0.57	1.32	55	42	33	43
Total iron (mg.)	34.58	22.02	12.83	23.16	—	—	—	—	—
Ionisable iron (mg.)	8.79	5.75	—	7.27	15.00	59	38	—	48
Copper (mg.)	1.85	5.27	2.58	3.23	—	—	—	—	—

DISCUSSION

The diets consumed in different seasons are significantly different. The diet in August is inferior in all the important ingredients studied to the diets in April and December. The mean consumption values for all the seasons show the following deficiencies in the students' dietaries when compared to the standards compiled by Stiebeling of the Government Bureau of Home Economics, U. S. A.

TABLE III

	Average consumption	Optimum requirement	Deficit
Protein ...	56.00 g.	75.00 g.	19.00 g.
Ether extract ...	37.43 g.	—	—
Calcium ...	0.75 g.	0.88 g.	0.13 g.
Available phosphorus ...	0.57 g.	1.32 g.	0.75 g.
Available iron ...	7.27 mg.	13 mg.	5.73 mg.

The students' dietaries are therefore particularly deficient in available phosphorus and available iron. Particular attention should therefore be given to meet these deficiencies and special attention should be paid in the monsoon during which the diets are most deficient. Most of the protein and calcium, however, are derived from vegetable sources which are of lower nutritional value. Students should be encouraged to consume animal foods such as milk, eggs, etc., which are very good sources of protein of high biological value and of available calcium and phosphorus.

To compare the figures obtained by the actual analysis of the cooked diets with those obtained by the survey method, a survey of the raw food-stuffs that were purchased and cooked in the different hostels was carried out during the period when their cooked diets were analysed. 10% of these figures was deducted for wastage in cooking, as is usually done. The figures for consumption obtained by the two methods are shown in Table IV, which showed in practically all cases a disparity between the figures.

TABLE IV

Daily consumption per individual calculated on raw food-stuffs basis compared with that obtained by analysis of the cooked food-stuffs consumed.

	Protein g.	Ether extract g.	Calcium g.	Phosphorus g.	Total iron mg.
HOSTEL No. 1					
Purchased food-stuff basis ...	79.65	63.65	1.25	1.43	16.5
Cooked diet analysis ...	65.72	49.83	0.66	0.90	27.6
HOSTEL No. 2					
Purchased food-stuff basis ...	73.5	41.18	1.50	1.06	11.6
Cook diet analysis ...	72.2	53.87	0.98	0.89	62.6
HOSTEL No. 3					
Purchased food-stuff basis ...	82.1	52.64	1.00	1.09	11.9
Cooked diet analysis ...	58.1	40.60	0.44	0.74	33.7

All the values except those for total iron obtained by the usual survey method are considerably higher than those obtained by the actual analysis of the cooked diets. This seems to indicate that the figures obtained by the usual survey method may be definitely misleading, and the method of analysis of sampled cooked diets, although more arduous, is to be preferred, whenever possible. The high value for iron in the cooked diet is perhaps to be explained by the universal use of iron vessels for cooking in Bengal, which practice, under the present circumstances, seems to be desirable.

SUMMARY

(1) Cooked diets of thirteen different students' hostels in Calcutta were analysed for protein, ether extract, calcium, total phosphorus, phytin phosphorus, total iron, ionisable iron and copper in three distinct seasons, namely, summer, monsoon and winter.

(2) The diets consumed in monsoon were more deficient in every respect than the diets in April and December, which themselves were partly deficient. Among the different hostels again, the diets in a girls' hostel and in two Moslem hostels were particularly deficient.

(3) The figures obtained by actual analysis of the cooked diet and those obtained by a survey of the raw food-stuffs that are purchased and cooked are discussed. The former method is preferred as the values obtained by the latter method may be misleading.

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2

BIOCHEMICAL OBSERVATIONS ON A NEW BACTERIUM ISOLATED FROM TAMARIND

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It is well-known that there is quite a large number of bacteria which are capable of decomposing organic matter into butyric acid along with other products. A new butyric acid bacterium of this type has been isolated from old tamarind.

METHOD OF ISOLATION

Some old tamarind was collected and a small portion of it was introduced into a number of flasks containing a medium consisting of 1% peptone, 2.5% glucose, and 1.5% CaCO₃ (excess) with the pH at 7.4. After seven days' incubation at 30-31°C the products of the flasks were examined and were found to contain butyric acid, butyl alcohol and traces of ethyl alcohol and lactic acid. No evolution of gas was detected in course of the reaction.

A small portion of the fermented liquid was taken in a sterile test tube containing 5 c.c. normal saline solution and the medium described above contained in several flasks was inoculated with it. After a few days the organisms were examined and many spores were observed. Since butyric acid bacteria, in general, are more heat-resisting, one of the flasks was heated for 5 minutes on a boiling water bath and a fresh medium was inoculated with it and incubated at 30-31°C. After a week it was examined and found to contain butyric acid, butyl alcohol, traces of ethyl alcohol and lactic acid. Plating was carried out from it on glucose-agar medium. After 24 hours' incubation a few types of colonies were observed. Each type was again plated for several times until a pure strain of each kind was obtained. With these pure organisms fresh media were inoculated and examined after a week and one of these types was found to be responsible for the production of butyric acid, etc. A stock culture of this type was made and kept for further experiments.

Description of the new bacterium.

1. Rods—mostly in pairs and some in short chains.
2. 4—6 μ × 1.0 μ when grown for 24 hours in glucose-agar medium.
3. Motile (18 hours old).
4. Gram positive.
5. Spores—oval, 2 × 1 μ. Terminal.
6. Agar slant—white, spreading, surface moist and shining.

7. Agar plate—Circular opaque centre, irregular margin, colony white, granular, moist.
8. Broth—A white precipitate at the bottom—solution clear.
9. Potato slant—Dry, creamy, slightly darkened and spreading.
10. Milk (litmus)—Acid, peptonised, no gas.
11. Indole—not formed.
12. Nitrate—reduced to nitrite.
13. Agar stab culture—no growth.
14. Gelatin—not liquefied.
15. Anærobic culture—no growth.
16. Action on carbohydrate and related substrates:

Substrate	Reaction
Glucose	Acid.
Sucrose	Acid.
Starch	Acid.
Glycerine	Acid (late, after 48 hours).
Dextrin	Acid (do)
Mannose	Acid.
Maltose	Acid.
Lactose	Acid.
Galactose	Acid (late, after 48 hours).
Arabinose	Acid (do).
Mannite	No acid.
Salicin	No acid.
Inulin	No acid.

No gas was evolved in any case.

17. Optimum pH—9.0.
18. Habitat—isolated from old tamarind.
19. Optimum temperature—35°-37°
20. Special characteristics—(1) no gas formation ; (2) liquid products are butyric acid, butyl alcohol, traces of ethyl alcohol and lactic acid.

The bacillus is morphologically different from *Clostridium butyricum* which also produces butyric acid and butyl alcohol by fermentation and it is definitely a different species. The important differences in its cultural characteristics from *Clostridium butyricum* are as follows :—

1. It is ærobic whereas *Cl. butyricum* is anærobic.
2. It gives no gas with carbohydrates, milk, potato etc., but *Cl. butyricum* produces CO_2 and H_2.
3. Litmus milk is peptonised, no coagulation—but *Cl. butyricum* coagulates milk with abundant gas formation.

Effect of heat on spores.
+ indicates growth.

Temp.			Time in minutes		
			5	10	15
100°C	...	...	+	+	+
110	...	...	+	+	+
115	...	...	+	+	+
120	..	...	+	+	no growth.

The spores are highly heat-resisting as is evident from the above table.

Effect of pH on fermentation.

pH	Glucose taken (g. in 100 c.c. medium).	Residual glucose (%)
6	5	40.6
7	,,	37.6
8	,,	37.4
9	,,	35.6
10	,,	37.2

From the above table it is clear that the optimum pH for the reaction is 9.0. No fermentation was observed at pH 5.0.

Effect of sugar concentration on the total fermentation.

G. of glucose per 100 c.c. of medium.	Residual glucose (%)
15	76.2
10	66.7
5	35.0

The largest percentage of fermentation of glucose was therefore obtained with a 5% conentration of glucose.

ACTION OF THE BACILLUS ON MOLASSES

In preliminary work the following results of the action of this bacillus on cane molasses were obtained.

% Butyl Alcohol.	% Butyric Acid.
22.39	36.1
24.85	34.1
25.10	33.0
23.75	35.0

It seems that under proper conditions 35% of butyric acid and 25% of butyl alcohol on the basis of the sugar in the molasses can be obtained by the action of the bacillus.

Summary

A new type of ærobic bacillus has been isolated from old tamarind, which can ferment glucose, sucrose, starch, glycerine, dextrin, mannose, maltose, lactose, galactose, arabinose but has no action on mannite, inulin and salicin. The optimum pH for the fermentation is 9.0. The growth of the bacteria is completely suppressed at pH 5. The spores of the bacteria are fairly heat-resisting. The products obtained by such fermentation are butyl alcohol, butyric acid and traces of lactic acid.

The bacillus differs from *Clostridium butyricum* in the following cultural characteristics:

It is ærobic ; it gives no gas with carbohydrates, milk and potato ; it peptonises litmus milk without coagulation.

Acting on molasses it can produce 35% butyric acid and 25% butyl alcohol on the basis of the sugar in the molasses under proper conditions.

THE EFFECT OF VITAMIN C AND CERTAIN OTHER SUBSTANC ON THE GROWTH OF MICRO-ORGANISMS

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The relation of vitamin C to the growth of plant organisms has attract attention for some time. Havas (1) observed that vitamin C in concentratio of 1/10,000 and 5/10,000 had an accelerating effect on the growth of whe seedlings but it had an inhibitory effect in a concentration of 2.5/1,00 and in larger concentrations it produced practically a lethal effec Hausen (2) observed that 40 mg. of ascorbic acid increased the dry weigh of treated pea plants to an extent of 35-75% in sterile culture experiment: Similar observations were made by Davis and his co-workers on the willow tomato and castor oil plants (3). Discussing Hausen's results, Virtanen (4 remarks that "it is reasonable to regard vitamin C as a phytohormone which is indispensable to plants."

Very little information, however, has been available regarding the effec: of vitamin C on micro-organisms, and we undertook this work in 1936, as it seemed to us that the study of the effect of vitamin C on such simple organisms might throw some light on the mechanism of the action of vitamin C. A note on preliminary results has been published (5). A few papers have, however, appeared subsequently bearing on the relation between vitamin C and certain bacteria (6-8). Some of this work is discussed later.

In the present work we have carried out some experiments on the effect of vitamin C on the growth of different fungi and bacteria in a synthetic medium. The cultures used were those of *Aspergillus niger*, *Asp. oryzæ*, *Asp. flavus*, *Saccharomyces cerevisiæ*, *S. ellipsoideus*, *B. subtilis*, *B. typhosus*, *B. coli*, *Ærobacter ærogenes*, *Staphylococcus aureus*, *Streptococcus hæmolyticus* and *B. diphtheria*.

METHOD

The synthetic medium of Reader (9) with slight modification was used in all the experiments. The inoculum throughout the experiments consisted of 0.1 c.c. of a suspension of a loopful of the culture (24 hours old) in 5 c.c. of sterile water. The estimation of growth was carried out by the determination of the dry weight in the case of *Aspergillus*, by counting with the hæmocytometer in the case of *Saccharomyces* and nephelometrically in the case of bacteria.

THE EFFECT OF VITAMIN C ON THE GROWTH OF FUNGI

At the beginning, in a few experiments different concentrations of ascorbic acid were used and 1/50,000 was found to have the maximum stimulating effect. A stronger concentration like 1/10,000 was, however, inhibitory to the growth of *Aspergillus* and *Saccharomyces*.

TABLE I

Effect of ascorbic acid on the growth of Asp. niger in different concentrations.

Figures give the wt. of Asp. niger in g. in 20 c.c. culture (72 hours old).

Concentration of ascorbic acid					
0	1/10,000	1/25,000	1/50,000	1/75,000	1/100,000
0.0113	0.0059	0.0087	0.0274	0.0162	0.0145
0.0153	0.0087	0.0130	0.0324	0.0246	0.0187
0.0072	0.0020	0.0053	0.0140	0.0108	0.0065

TABLE II

Effect of ascorbic acid on the growth of S. cerevisiæ in different concentrations.

Figures indicate no. of cells per c.c. in thousands.

Concentration of ascorbic acid					
0	1/10,000	1/25,000	1/50,000	1/75,000	1/100,000
600	250	450	4250	2400	1200
100	—	60	2150	800	275
225	75	130	3000	1700	500

With all the varieties of *Aspergillus*, the addition of vitamin C (1/50,000) to the medium resulted in very markedly increased growth during the first 72 hours ; after five days, however, the total growth was approximately equal to that in the flask in which no ascorbic acid or ascorbic acid, which had been previously oxidised irreversibly, had been added.'

TABLE III

Wt. of Aspergillus in g. in 20 c.c. culture.

	72 hours old culture			120 hours old culture		
	Without ascorbic acid	With ascorbic acid 0.4 mg.	With ascorbic acid (oxidised) 0.4 mg.	Without ascorbic acid	With ascorbic acid 0.4 mg.	With ascorbic acid (oxidised) 0.4 mg.
Asp. niger.	0.0128	0.0222	—	0.0320	0.0304	—
	0.0168	0.0261	—	0.0356	0.0369	—
	0.0098	0.0179	—	0.0250	0.0284	—
	0.0072	0.0139	—	0.0235	0.0229	—
	0.0130	0.0302	—	0.0342	0.0359	—
	0.0087	0.0127	—	0.0223	0.0235	—
	0.0090	0.0158	—	0.0240	0.0252	—
	0.0153	0.0324	0.0129	0.0346	0.0360	0.0353
	0.0122	0.0335	0.0132	0.0348	0.0382	0.0329
	0.0104	0.0170	0.0118	0.0256	0.0242	0.0249
	0.0059	0.0202	0.0043	0.0219	0.0232	0.0231
	0.0113	0.0274	0.0103	0.0325	0.0342	0.0318
Asp. oryzæ.	0.0085	0.0234	0.0079	—	—	—
	0.0125	0.0402	0.0140	—	—	—
	0.0042	0.0180	0.0030	—	—	—
	0.0027	0.0098	0.0037	—	—	—
	0.0011	0.0063	0.0020	—	—	—
	0.0032	0.0084	0.0027	0.0285	0.0301	0.0278
	0.0015	0.0039	0.0020	0.0164	0.0153	0.0149
	0.0044	0.0140	0.0035	0.0364	0.0410	0.0338
Asp. flavus.	0.0014	0.0031	—	—	—	—
	0.0017	0.0049	—	—	—	—
	0.0012	0.0042	—	—	—	—
	0.0009	0.0027	—	—	—	—
	0.0015	0.0039	—	—	—	—
	0.0023	0.0052	—	—	—	—
	0.0010	0.0034	0.0017	0.0072	0.0085	0.0063
	0.0020	0.0057	0.0024	0.0123	0.0109	0.0132
	0.0008	0.0030	0.0015	0.0098	0.0104	0.0083
	0.0018	0.0043	0.0012	0.0102	0.0115	0.0121

From Table III it will be noticed that the growth was simply stimulated by the addition of ascorbic acid and maximum growth was attained

earlier than in the controls ; controls reached the maximum in five days, the final growth being approximately the same in both sets of experiments. Experiments were carried out with other reducing agents such as glutathione and cysteine to find how far the action of ascorbic acid is specific. From Table IV it will be seen that, though ascorbic acid has a greater stimulating action than the other two reducing substances, the latter stimulate growth quite markedly.

TABLE IV.

Figures indicate wt. of Aspergillus in g. in 20 c.c. culture.

	72 hours old culture				120 hours old culture.			
	Control	Glutathione 0.4 mg.	Cysteine 0.4 mg.	Ascorbic acid 0.4 mg.	Control	Glutathione 0.4 mg.	Cysteine 0.4 mg.	Ascorbic acid 0.4 mg.
Asp. niger.	0.0098	0.0153	0.0130	0.0250	0.0255	0.0263	0.0272	0.0285
	0.0128	0.0250	0.0186	0.0287	0.0348	0.0327	0.0310	0.0382
	0.0072	0.0163	0.0116	0.0210	0.0160	0.0126	0.0147	0.0180
	0.0059	0.0130	0.0096	0.0178	0.0384	0.0326	0.0284	0.0344
Asp. flavus.	0.0024	0.0067	0.0053	0.0114	0.0224	0.0240	0.0186	0.0238
	0.0031	0.0083	0.0064	0.0130	0.0253	0.0223	0.0198	0.0244
	0.0012	0.0058	0.0030	0.0082	0.0146	0.0106	0.0090	0.0132
	0.0015	0.0072	0.0043	0.0092	0.0160	0.0176	0.0138	0.0153
Asp. oryzæ.	0.0044	0.0096	0.0084	0.0134	0.0186	0.0170	0.0153	0.0206
	0.0035	0.0045	0.0039	0.0118	0.0179	0.0163	0.0140	0.0198
	0.0027	0.0053	0.0047	0.0110	0.0163	0.0168	0.0119	0.0184
	0.0042	0.0076	0.0058	0.0140	0.0210	0.0190	0.0176	0.0225

SYNTHESIS OF ASCORBIC ACID BY FUNGI

Several experiments were conducted to see whether the fungi are themselves capable of synthesising ascorbic acid. From Table V it will be observed that in all the cases the organisms were capable of synthesising vitamin C. In the experiments where glutathione and cysteine were used, an appreciably larger quantity of ascorbic acid was formed. The estimation of ascorbic acid was carried out with the trichloroacetc acid extracts of the micro-organisms by titration with 2:6-dichlorophenol indophenol in the usual way.

TABLE V.

Figures indicate mg. of ascorbic acid formed in 200 c.c. culture

	72 hours old culture.				120 hours old culture.			
	Control	Glutathione 4.0 mg.	Cysteine 4.0 mg.	Ascorbic acid 4.0 mg.	Control	Glutathione 4.0 mg.	Cysteine 4.0 mg.	Ascorbic acid 4.0 mg.
Aspergillus niger.	0.146	0.208	0.183	0.246	0.376	0.253	0.208	0.353
	0.160	0.198	0.179	0.287	0.432	0.349	0.324	0.418
	0.153	0.176	0.164	0.210	0.353	0.276	0.246	0.326
	0.124	0.146	0.130	0.186	0.298	0.240	0.221	0.304
Aspergillus oryzæ.	0.127	0.168	0.156	0.186	0.208	0.186	0.173	0.230
	0.116	0.130	0.117	0.167	0.178	0.159	0.140	0.286
	0.104	0.148	0.130	0.159	0.174	0.168	0.152	0.280
	0.122	0.153	0.143	0.193	0.221	0.208	0.189	0.243
Aspergillus flavus.	0.091	0.146	0.140	0.173	0.198	0.189	0.160	0.208
	0.104	0.160	0.144	0.208	0.208	0.190	0.183	0.221
	0.078	0.124	0.104	0.140	0.176	0.151	0.140	0.188
	0.083	0.153	0.130	0.168	0.182	0.194	0.156	0.198

With *Saccharomyces* also the addition of ascorbic acid markedly stimulated the growth, with the only difference that the growth was always greater than that in the negative controls even after five days (Table VI).

TABLE VI.

Effect of ascorbic acid on the growth of Saccharomyces cerevisiæ (after 72 hours) in 20 c.c. culture

Expt. no.	Without ascorbic acid. Number of cells per c.c. in thousands.	With ascorbic acid 0.4 mg. Number of cells per c.c. in thousands.	With ascorbic acid (oxidised) 0.4 mg. Number of cells per c.c. in thousands.
1.	600	4240	—
2.	700	3800	—
3.	660	4600	—
4.	600	4000	—
5.	600	4500	—
6.	400	2900	—
7.	700	4500	—
8.	100	840	—
9.	800	3600	—
10.	600	2720	—
11.	900	2126	—
12.	500	1600	560
13.	650	6050	440
14.	780	4650	840
15.	225	1204	350
16.	600	2060	450
17.	400	2600	530
18.	700	3200	590
19.	100	1700	230

Effect of ascorbic acid on the growth of Saccharomyces ellipsoideus in 20 c.c. culture (after 72 hours)

Expt. no.	Without ascorbic acid. Number of cells per c.c. in thousands.	With ascorbic acid 0.4 mg. Number of cells per c.c. in thousands.	With ascorbic acid (oxidised) 0.4 mg. Number of cells per c.c. in thousands.
1.	660	4000	—
2.	700	3200	—
3.	400	6600	—
4.	1380	3600	—
5.	900	2700	—
6.	800	3600	—
7.	1000	4600	—
8.	1200	4000	—
9.	600	4240	—
10.	700	3800	—
11.	660	4000	450
12.	1400	4500	1250
13.	600	2940	840
14.	400	1050	450
15.	700	3200	610

Similar experiments with glutathione and cysteine were carried out and gave similar results as with ascorbic acid (Table VII).

TABLE VII.

Effect of ascorbic acid, glutathione, cysteine on the growth of Saccharomyces

Figures indicate no. of cells per c.c. in thousands.

	Control	Glutathione 0.4 mg. in 20 c.c. culture.	Cysteine 0.4 mg. in 20 c.c. culture.	Ascorbic acid 0.4 mg. in 20 c.c. culture.
Saccharomyces cerevisiæ.	600	3210	1570	4250
	225	760	500	1250
	400	1850	900	2900
	100	1250	450	2000
	500	3200	1250	3600
Saccharomyces ellipsoideus.	400	800	580	1050
	660	2500	1070	4000
	800	2800	1200	3600
	700	2500	1500	3800
	600	2100	1350	3000

Parallel estimations of the vitamin C content of the cells have shown that it increases with the increasing growth which will be seen from Table VIII.

TABLE VIII.

Wt. of ascorbic acid (mg.) formed in 200 c.c. Saccharomyces culture

	72 hours old culture.				120 hours old culture.			
	Control	Glutathione 4.0 mg.	Cysteine 4.0 mg.	Ascorbic acid 4.0 mg.	Control	Glutathione 4.0 mg.	Cysteine 4.0 mg.	Ascorbic acid 4.0 mg.
S. cervisiæ.	0.025	0.059	0.046	0.186	0.062	0.188	0.139	0.386
	0.038	0.078	0.062	0.223	0.091	0.268	0.198	0.448
	0.032	0.081	0.049	0.195	0.087	0.244	0.178	0.462
S. ellipsoideus.	0.072	0.146	0.120	0.296	0.188	0.264	0.203	0.524
	0.048	0.078	0.096	0.136	0.125	0.185	0.164	0.329
	0.104	0.160	0.129	0.208	0.230	0.319	0.288	0.582

THE EFFECT OF VITAMIN C ON THE GROWTH OF BACTERIA

The proliferation of bacteria like *B. subtilis, B. typhosus, B. coli, Aerobacter aerogenes, Staphylococcus aureus, Streptococcus hæmolyticus* and *B. diphtheria,* appeared to be inhibited instead of stimulated by ascorbic acid.

In these cases also (except with the last two bacteria mentioned above) ascorbic acid of strength 1/50,000 was found to give the best inhibitory result. Ascorbic acid of higher dilution also had inhibitory action (Table IX).

TABLE IX.

Effect of ascorbic acid on the growth of different bacteria in different concentrations.

Figures indicate no. of cells per c.c. in millions.

	Concentration of ascorbic acid					
	0	1/10,000	1/25,000	1/50,000	1/75,000	1/100,000
B. subtilis.	757	—	—	—	190	569
	1136	—	—	—	190	757
	379	—	—	—	—	190
B. typhosus.	1373	—	—	—	229	915
	686	—	—	—	170	458
	915	—	—	—	229	686
—	1543	—	—	—	229	1373
Staphylococcus aureus.	379	—	—	—	—	190
	379	—	—	—	slight turbidity	190
	758	—	—	—	190	569

*In this and subsequent Tables (—) indicates no recognisable growth.

Glutathione and cysteine in concentrations of 1/50,000 also have some inhibitory effect on the growth of the bacteria studied (Table X).

TABLE X.

Figures indicate no. of cells per c.c. in millions.

	Control.	Glutathione.	Cysteine.	Ascorbic Acid.
	757	—	190	—
	379	—	—	—
	379	—	—	—
	757	—	379	—
	379	—	—	—
	1136	379	379	—
B. coli.	757	190	379	—
	379	—	—	—
	757	—	—	—
	1136	379	379	—
	379	—	—	—
	379	—	—	—
	757	—	—	—
	1137	190	379	—
	758	—	190	—
Staphylococcus aureus.	570	—	—	—
	758	—	190	—
	379	—	—	—
	1327	570	570	—
	458	—	—	—
	915	229	229	—
	915	458	458	—
B. typhosus.	686	458	458	—
	915	229 (below)	229	—
	458	—	—	—
	1373	458	458	—
	379	—	—	—
	379	—	—	—
	757	—	379	—
	1136	379	379	—
	757	190	379	—
	379	—	—	—
	1136	379	759	—
	379	—	—	—
	757	—	379	—
	757	—	—	—
B. subtilis.	1136	379	759	—
	757	—	—	—
	379	—	—	—
	379	—	—	—
	759	—	—	—
	1136	379	379	—
	379	—	—	—
	1136	759	759	—

TABLE X (*contd.*)

	757	—	—	—
	379	—	—	—
	379	—	—	—
	1136	—	190	—
	379	—	—	—
	379	—	—	—
	379	—	—	—
	379	—	—	—
Aerobacter	757	—	379	—
aerogenes.	1136	190	379	—
	379	—	—	—
	379	—	—	—
	757	—	—	—
	1136	379	379	—
	379	—	—	—
	757	—	—	—
	1136	379	379	—
	757	—	379	—

In much lower concentrations of vitamin C viz. in 1/100,000, there is no inhibitory effect, but there is no stimulating effect either. In this respect the fungi therefore behaved very differently from the bacteria.

Kodama and Kojima (10) have also observed an inhibitory effect of vitamin C on the growth of *Staphylococcus* cultures but Farber states that he found a stimulating action of vitamin C on the growth of *Staph. aureus.* We, therefore, reinvestigated the question by using the casein digest medium of Farber and confirmed our finding obtained with Reader's medium regarding the inhibitory effect of vitamin C on *Staph. aureus* (Table XI). The casein digest medium used by us was as follows:

Tryptic digested casein 1.0%, NaCl 1.0%, K_2HPO_4 0.2%, water 100 c.c., *p*H adjusted to 7.2—7.4.

TABLE XI.

Effect of ascorbic acid on the growth of Staph. aureus

Figures indiciate no. of cells per c.c. in millions

Reader's medium.		Tryptic digested casein.		
Control.	1/50,000	Control.	1/50,000	1/20,000
758	—	758	678	379
379	—	1516	1516	678
285	—	1895	1516	758
190	—	758	678	190
379	—	1137	758	379

THE EFFECT OF VITAMIN C ON THE GROWTH OF B. DIPHTHERIA AND
STREPTOCOCCUS HÆMOLYTICUS

As the two organisms *B. diphtheria* and *Streptococcus hæmolyticus* did not grow well in Reader's medium, the following medium was used:

Beef extract	...	0.3%
Peptone	...	1%
Sodium chloride	...	0.5%
Glucose	...	0.5%
Glycerine	...	2.0%

With these two organisms also vitamin C had an inhibitory effect in a concentration of 1/20,000. With 1/50,000 concentration the inhibitory effect is somewhat less (Table XII).

TABLE XII.

The effect of ascorbic acid on the growth of B. diphtheria and Streptococcus hæmolyticus

Figures indicate no. of cells per c.c. in millions

	Concentration of ascorbic acid		
	o	1/50,000	1/20,000
B. diphtheria.	403	202	—
	605	403	—
	1210	403	—
	202	—	—
	806	202	—
	1613	1210	202
Streptococcus hæmolyticus.	994	331	—
	663	331	—
	165	—	—
	331	—	—
	994	165	—
	1157	663	—
	331	165	—

EFFECT OF NICOTINIC ACID, ADRENALIN AND INSULIN ON THE GROWTH
OF B. SUBTILIS, B. DIPHTHERIA AND SACCH. CEREVISIÆ

It was considered of interest to investigate what effect another vitamin like nicotinic acid and hormones available in the pure form like adrenalin and insulin would have on the growth of typical bacteria and fungi. For bacteria, *B. subtilis* and *B. diphtheria* were used, the former grown in Reader's medium and the latter in the medium indicated above. *Sacch. cerevisiæ* was used as a typical fungus and it was grown in Reader's medium. The addenda were all used in 1/50,000 concentration, except in the case of B. diphtheria, where it was used in 1/20,000 concentration. The results (Table XIII) shows that in all cases there was a stimulation of the growth of the organisms, both bacteria and fungi. In this respect these addenda differ from ascorbic acid which in the same concentration

stimulates the growth of fungi only. Hormone preparations like Antuitrin S, Antuitrin G and Pitressin (Parke Davis & Co.) had also a stimulating effect on the above micro-organisms in a dilution of 1/50,000, but no significance can be attached to these results as the hormones concerned have not yet been obtained in a chemically pure state. The effect of adrenalin, insulin and nicotinic acid deserves further investigation in order to find whether it is merely due to provision of extra nitrogenous material as a nutriment or due to some specific stimulation.

EFFECT OF ASCORBIC ACID ON THE GROWTH OF A SMALL INOCULUM OF SACCH. CEREVISIÆ AND STAPH. AUREUS

It was considered desirable to carry out two typical experiments, one with a fungus and another with a bacterium, with a very small and definite number of cells in the inoculum to start with and counting the cells at the end of a stated period of incubation. These results obtained with *Sacch. cerevisiæ, Sacch. ellipsoideus* and *Staph. aureus* are given in Tables XIV (a) and (b) ; they confirm the previous results regarding the action of ascorbic acid on bacteria and fungi.

TABLE XIII.
Figures indicate no. of cells per c.c. in millions

	Control.	Ascorbic Acid.	Antuitrin S.	Antuitrin G.	Adrenalin chloride	Nicotinic acid.	Insulin.	Pitressin.
	379	—	379	757	569	757	1136	1136
	190	—	379	379	379	757	757	1136
	379	—	757	757	379	569	757	757
B. subtilis.	379	—	379	757	757	757	569	757
	379	—	757	757	757	1136	1136	1136
	379	—	757	569	757	757	569	569
	190	—	379	379	757	379	757	757
	190	—	379	379	379	757	757	757
	202	—	403	403	403	806	1210	1210
	403	—	605	806	806	1613	1210	1613
	403	202	806	1210	806	1210	1613	2016
B. diphtheria.	202	—	403	605	605	806	403	806
	403	—	806	1210	806	806	1210	1613
	202	—	403	806	605	403	403	806
	202	—	403	806	403	806	806	806
	202	—	605	806	403	806	403	1210
Figures indicate no. of cells per c.c. in thousands								
	320	2900	656	1224	800	1150	1500	1800
	280	1250	608	832	732	800	900	1100
	560	3000	672	664	664	925	1100	1560
Sac. cerevisiæ.	600	4250	696	774	724	1025	1500	2250
	400	2900	900	1150	925	1225	1500	2000
	225	1250	560	700	600	725	780	950
	500	3600	760	1050	825	1300	1625	2500

TABLE XIV (a)

	Number of cells per 0.1 c.c. inoculum.	Number of cells per c.c. of culture in thousands.			
		Control.	Glutathione. 1/50,000	Cysteine. 1/50,000	Ascorbic acid. 1/50,000
Sacch. cerevisiæ.	50	600	3240	1445	4050
	25	400	925	580	1625
	23	225	625	500	1050
Sacch. ellipsoideus.	20	400	850	575	1050
	32	660	2500	1070	4000
	15	275	600	400	800

TABLE XIV (b)

	No. of cells per 0.1 c.c. inoculum.	No. of cells per c.c. in millions after 72 hours' incubation.	
		Control (without ascorbic acid).	With ascorbic acid.
Staph. aureus.	180	450	—
	240	796	—
	115	360	—

The cocci were counted by proportionate counting method.

DISCUSSION

Although considerable work has been carried out in various laboratories to explore the mechanism of action of vitamin C in the body and some important facts have been discovered, a connected picture of the various aspects of its rôle in life is not yet possible. It was considered that an investigation on the effect of vitamin C on primitive unicellular organisms might throw some light on the fundamental mode of action of vitamin C. Some curious results have been obtained. Thus in concentrations of 1/50,000, vitamin C has a stimulating action on the growth of the fungi studied but an inhibitory action on that of bacteria. If the concentration of vitamin C is increased to 1/10,000, the growth of the fungi is also inhibited. At the same time the fungi themselves have the power to synthesise vitamin C, so that it would seem that although vitamin C is needed for their growth, given the time, they can themselves synthesise it. If, however, it is added in a preformed condition at the start of the incubation, the growth of the cells is hastened, though in course of 5 days or so the growth of these and of the controls is largely equalised owing to the synthesis of vitamin C by the latter. In trying to investigate the specificity of this action of vitamin C, other naturally occurring reducing substances like glutathione and cysteine were also found to stimulate the action of vitamin C though to a less marked degree. Similarly these reducing agents also inhibited the

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growth of certain bacteria like vitamin C. It would thus seem probable that the action of vitamin C with reference to the growth of fungi is associated with some sort of reducing action and that the agent concerned in the natural process of proliferation is vitamin C, whose redox potential and other characteristics are perhaps more suitable for the complicated bio-chemical mechanism underlying the cell-division of the fungi than glutathione or cysteine, which simulates the action of the vitamin to some extent. It is possible that the same properties of vitamin C adversely affect the growth of bacteria in 1/50,000 concentration and that of fungi in 1/10,000 concentration. Beyond this, it is not possible to speculate at the present stage.

SUMMARY

(1) Ascorbic acid has got a stimulating effect on the growth of the following fungi—*Aspergillus niger, Asp. oryzæ, Asp. flavus, Sach. cerevisiæ, S. ellipsoideus,* in a synthetic medium in a concentration of 1/50,000 and an inhibitory effect in a concentration of 1/10,000.

(2) Ascorbic acid has got an inhibitory effect on the growth of the following bacteria—*B. subtilis, B. typhosus, B. coli, Aerobacter aerogenes, Staphylococcus aureus, Streptococcus hæmolyticus* and *B. diphtheria,* in a synthetic medium in a concentration of 1/50,000 except in the last two cases, which require a concentration of 1/20,000. In concentration of 1/100,000 vitamin C has neither any stimulating nor any inhibitory action on these bacteria.

(3) The above effect of vitamin C is simulated by other reducing substances like glutathione and cysteine though to a less marked degree.

(4) In the case of certain fungi, it has been found that they can synthesise their own ascorbic acid. The addition of ascorbic acid at the start of the incubation apparently produces stimulation of growth during the first two or three days, which later becomes roughly equal to that of the controls owing to the latter synthesising their own vitamin C.

(5) Nicotinic acid, adrenalin and insulin have a stimulating action on the growth of bacteria and fungi in 1/50,000 concentration but significance regarding specificity may not be attached to these results at present.

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URINARY EXCRETION OF COMBINED ASCORBIC ACID IN PULMONARY TUBERCULOSIS

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(Received on July 12, 1940)

It has been shown earlier (1) that a number of plant tissues contain ascorbic acid in a combined state (ascorbigen) besides free ascorbic acid. Further work has confirmed this view and it has lately been possible to effect considerable concentration of ascorbigen from cabbage (2). The existence in normal urine of ascorbic acid in combined state was indicated by Scarborough and Stewart (3) and ourselves (4) but there are reasons to believe that the urinary substance or substances are not identical with the ascorbigen of plant tissues. Work in this laboratory during the last few years has indicated the possibility that ascorbic acid may function as a detoxicating agent in the normal condition as also in infected conditions and it has been shown that the injection of diphtheria and tetanus toxins into guinea-pigs is followed by increased urinary excretion of combined ascorbic acid (5). Abbasy, Hill and Harris (6) also observed a diminution in the urinary excretion of free ascorbic acid in certain infected conditions. It is possible that some of the disappearing ascorbic acid is destroyed by increased metabolism in the infected condition but no systematic work on the fate of the disappearing ascorbic acid seems to have been undertaken. In an effort to throw light on this question, we have estimated the relative excretion of free ascorbic acid, dehydro-ascorbic acid and combined ascorbic acid of nine normal healthy adults and of sixteen patients suffering from acute pulmonary tuberculosis. A note on this work has been published elsewhere (7).

The urine was collected in an amber coloured bottle containing sulphuric acid. The concentration of sulphuric acid used to preserve the ascorbic acid in urine was 5 per cent by volume. This has acted as a very good preservative, as has been shown by us elsewhere (8). In actual experiments, 100 c.c. of concentrated sulphuric acid mixed with 100 c.c. of water were placed in a Winchester amber-coloured bottle considering that the total volume of urine excreted during 24 hours would be roughly 1800 c.c. Aliquots of this collected urine obtained after twenty-four hours from each individual were examined for (i) free ascorbic acid, (ii) dehydro-ascorbic acid and (iii) combined ascorbic acid. The methods for their estimation are indicated below.

Estimation of free ascorbic acid : Urine was titrated against 2:6-dichlorophenol indophenol in the usual way (4). An aliquot of the urine was treated with sufficient ascorbic acid oxidase (prepared from cucumber) to oxidise all the ascorbic acid present and titrated against the

indophenol dye. The difference between the titration values before and after oxidase treatment gave the 'true' free ascorbic acid value.

Estimation of dehydro-ascorbic acid : An aliquot portion of the urine was taken and hydrogen sulphide was passed for 20 min. Hydrogen sulphide was chased off by a current of carbon dioxide. An aliquot was titrated against the indophenol reagent, and another portion was treated with ascorbic acid oxidase as before. The difference between the titration values before and after the oxidase treatment gave the 'true' total. value of free and dehydro-ascorbic acid. Deducting the value of true free ascorbic acid from the above difference, the value of 'true' dehydro-ascorbic acid was obtained.

Estimation of combined ascorbic acid : Into a portion of urine hydrogen sulphide was passed for 5 minutes to drive out any dissolved oxygen. The container was then placed in a water-bath and hydrogen sulphide was passed for another 10 minutes. The container was cooled and hydrogen sulphide was passed for another 5 minutes. This treatment with hydrogen sulphide in the hot condition reduced the dehydro-ascorbic acid and split up the combined ascorbic acid. Hydrogen sulphide was chased off by a current of carbon dioxide. One aliquot was titrated with indophenol as before and another was treated with ascorbic acid oxidase. The difference between the titration values gave the value for 'true total' ascorbic acid. Subtracting the values of free and dehydro-ascorbic acid from the above result the combined ascorbic acid value was obtained.

Tables I and II give the values for urinary ascorbic acid in tuberculous and normal conditions.

TABLE I.

Excretion of ascorbic acid in pulmonary tuberculosis in 24 hours' urine.
(Figures are expressed in terms of free ascorbic acid in mg.)

Patient no.	Free ascorbic acid	Dehydro-ascorbic acid	Combined ascorbic acid	Total ascorbic acid.	Combined as % of total
1.	7.19	2.08	5.73	15.00	38.2
2.	3.89	1.97	3.20	9.06	35.3
3.	6.72	2.68	4.36	13.76	31.7
4.	4.32	3.11	7.98	15.40	51.8
5.	6.27	8.27	nil	14.54	nil
6.	7.86	0.29	3.85	12.00	32.1
7.	1.98	1.98	6.68	10.64	62.8
8.	7.82	1.60	16.27	25.49	63.8
9.	4.55	0.93	12.07	17.55	68.8
10.	2.81	9.05	4.20	16.46	26.2
11.	7.20	8.00	14.26	29.46	48.4
12.	6.72	3.40	10.21	20.33	50.2
13.	4.61	2.69	26.10	33.40	78.1
14.	6.15	4.18	1.07	11.40	9.4
15.	9.28	4.57	6.49	20.34	31.9
16.	5.70	2.52	7.31	15.53	47.1
Mean	5.82	3.52	8.11	17.45	42.24

TABLE II.

Excretion of ascorbic acid in normal individuals in 24 hours' urine.
(Figures are expressed in terms of free ascorbic acid in mg.)

Subject no.	Free ascorbic acid	Dehydro-ascorbic acid	Combined ascorbic acid	Total ascorbic acid	Combined as % of total
1.	31.70	5.45	22.40	59.55	37.6
2.	26.60	1.79	10.29	38.68	26.6
3.	28.69	nil	11.58	40.27	28.8
4.	48.37	25.50	27.80	101.67	27.3
5.	39.90	15.28	32.82	88.00	37.3
6.	67.11	14.11	3.22	84.44	3.8
7.	29.43	nil	7.39	36.82	20.1
8.	71.42	4.53	19.77	95.72	20.7
9.	58.39	nil	8.15	66.54	12.2
Mean	44.62	7.41	15.93	67.96	23.82

A statistical analysis of the above results was kindly carried out by Mr. K. C. Basak. On an average the proportion of combined ascorbic acid excretion in tuberculosis patients is 42.24 per cent of the total excretion while in normal healthy individuals it is only 23.82 per cent. In other words, the rate of excretion of combined ascorbic acid in tuberculous patients is nearly double the rate in healthy persons.

On testing the significance of the difference between the two mean rates, the following results are obtained.

$$\begin{aligned} \text{Difference of mean rates} &= 18.42 \\ \text{Standard error of difference} &= 7.56 \\ t &= 2.44 \end{aligned}$$

The possibility of obtaining such values of t by chance alone is less than five in hundred. Hence the proportion of combined ascorbic acid excreted is significantly higher in tuberculous patients than in normal healthy individuals.

DISCUSSION AND SUMMARY

As mentioned above several observers have noted a diminution of the excretion of ascorbic acid in certain infected conditions. It is probable that the disappearance of some of the vitamin is due to its oxidation by increased metabolism caused by infections. It appears, however, from the present results that some of the vitamin is excreted in a combined state thereby causing a diminution in the observed excretion of free ascorbic acid. In the estimation of ascorbic acid, dehydro-ascorbic acid and combined ascorbic acid described in this paper, ascorbic acid oxidase was always used in order to make the chemical method of determination of ascorbic acid more

specific.   The view has been advanced from this laboratory that vitamin C may have a detoxicating function both in normal and in infected conditions by combining with toxins or toxic metabolites and eliminating them.   The results described in this paper would appear to lend support to this view in reference to acute pulmonary tuberculosis and in this condition therefore and presumably in other infected conditions, the administration of massive doses of ascorbic acid is likely to give beneficial results.

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ENZYMES IN SNAKE VENOM

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Investigation on the enzymic activity of the venoms of many poisonous snakes has revealed the existence of a large number of enzymes such as proteinase, esterase, phosphatase, oxidase, etc. in them. The proteolytic activity of some of the venoms has been investigated by Flexner and Nóguchi (1), Delezenne (2), Noc (3), Lannoy (4), Housey and Negrete (5), Ghosh (6) and others. The esterase activity has been studied recently by the authors and by Iyengar and co-workers (7), the phosphatase activity by Uzawa (8) and by Gulland and Jackson (9). The present authors undertook a systematic study of the various enzymes which occur in the venom of a number of poisonous snakes which are common in India. The results obtained are recorded in this paper.

It has been recorded by several early investigators that the venoms of many snakes can exert marked digestive action on animal tissues. Flexner and Noguchi, Delezenne, Noc, Lannoy and others observed that snake venom can hydrolyse proteins like gelatin, egg albumen, casein etc. Lannoy showed that the proteolysis stops when albumose is formed and does not proceed far enough to form peptone. The influence of the reaction of the medium on the proteolytic activity of snake venom was not studied by the early workers. It is now recognised that the proteinase of animal origin can be classified in three groups, based on their activity in media of different pH.

  (*i*) A proteinase which is active in distinctly acid range is called pepsin.
  (*ii*) That which is active in slightly alkaline range with an optimum at about pH 8.0 is called trypsin.
  (*iii*) That which shows optimum activity in the neighbourhood of pH 5 is called kathepsin.

With a view to determine the nature of the proteinase in venom, we studied its activity at different pH from 2.0 to 9.4. The venoms used were those of Cobra (Naja Naja), Vipera Russellii, Banded Krait (B. Fasciatus) and Echis Carinata and the substrates used were casein, egg albumen and electrodialysed gelatin.

5 per cent solutions of egg albumen and gelatin were prepared in physiological saline and a 5 per cent solution of casein was prepared by dissolving it in 0.4 per cent sodium bicarbonate solution. 1 per cent solutions of the venoms were prepared in physiological saline shortly before use. The extent of hydrolysis was followed by Willstätter's method of

titration in 90 per cent alcoholic solution with alcoholic caustic potash, using thymolphthalein as an indicator. The pH of the reaction media was maintained nearly constant by adding buffer solutions. Between pH 2 and pH 5 citrate buffers were used and between pH 6 and 9.6 phosphate and borate buffers were used. In a series of flasks were placed 7 c.c. of the substrate solution adjusted to requisite pH, 5 c.c. of buffer solution of the same pH, 2 c.c. of the solution of venom and 6 c.c. of physiological saline. In the control flasks were placed the same volumes of the respective solutions but the solutions of the venoms were previously heated at 75° for about 50 minutes to destroy the proteinase in them. To each of the flasks a few drops of toluene were added to prevent bacterial growth and they were placed in a thermostat at 35°C. After suitable intervals of time 5 c.c. portions of the solutions were withdrawn and added to 45 c.c. of alcohol and immediately titrated with alcoholic caustic potash solution using thymolphthalein as indicator. Results obtained with gelatin as substrate are only recorded in Table I. It will be noticed that the proteinase in the venoms used has its optimum activity in the neighbourhood of pH 8. With casein as substrate the optimum activity was found to be in the neighbourhood of pH 7.4. These results suggest that the proteinase in the venom of the four different varieties of snake is very probably trypsin.

TABLE I.
Venom—Naja Naja.
0.04 N KOH required in c.c. to titrate 5 c.c. of solution.

pH	Venom + Substrate	Control	Diff.
2.0	5.60	5.60	0.00
4.0	3.40	3.40	0.00
5.0	2.85	2.80	0.05
6.0	2.68	2.48	0.20
7.0	2.40	1.95	0.45
7.4	2.28	1.80	0.48
7.8	2.10	1.50	0.60
8.4	1.82	1.22	0.60
9.0	1.54	1.12	0.42

Venom—Russell's Viper.
0.045 N KOH required in c.c. to titrate 5 c.c. of solution.

pH	Venom + Substrate	Control	Diff.
4.8	2.75	2.64	0.11
5.8	2.64	2.46	0.18
6.6	2.40	2.15	0.25
7.0	2.30	2.00	0.30
7.6	2.15	1.70	0.45
8.0	1.90	1.40	0.50
8.6	1.62	1.18	0.44
9.0	1.48	1.08	0.40

Venom—Echis Carinata.

0.0366 N caustic potash required in c.c. to titrate 5 c.c. solution.

pH	Venom + Substrate	Control	Diff.
5.0	2.88	2.81	0.07
6.0	2.66	2.44	0.22
6.5	2.49	2.11	0.38
7.0	2.38	1.91	0.47
7.6	2.21	1.70	0.51
8.0	1.95	1.41	0.54
8.5	1.72	1.22	0.50
9.0	1.52	1.10	0.42

Venom—B. Fasciatus.

*0.0452 N alcoholic caustic potash required in c.c. to titrate
5 c.c. solution.*

pH	Venom + Substrate	Control	Diff.
6.2	2.48	2.10	0.38
7.2	2.23	1.58	0.65
7.6	2.22	1.42	0.80
8.2	2.13	1.20	0.93
9.0	1.61	1.00	0.61

THE CRITICAL INACTIVATION TEMPERATURE OF THE PROTEINASE IN SNAKE VENOM:

The temperature at which the solutions of the different venoms lose one-half of their original proteolytic activity when heated for one hour was also determined. This critical inactivation temperature, although it varies somewhat with the protein content of an enzyme preparation, is of much help in establishing the identity of an enzyme. Therefore, a series of solution of the venoms were prepared and heated at the desired temperature for one hour. They were then quickly cooled under a tap and a fixed quantity (2 c. c.) was withdrawn from each solution and allowed to act separately on 18 c.c. of an 1.9 per cent. solution of casein mixed with phosphate buffer at pH 7.2. They were then kept in a thermostat at 35°C and the extent of hydrolysis was determined by withdrawing 5 c. c. of solutions at suitable intervals and titrating it by Sorensen's method. The results are recorded in Table II. It will be noticed from these data that the critical inactivation temperatures of the proteinase in the venoms of Naja Naja, B. Fasciatus, Echis Carinata and Vipera Russellii are 53°, 55°, 55° and 62° respectively while that of trypsin (E. Merck) is 50° and that of trypsin dissolved· in previously heated Cobra venom solution is 60°. It may be mentioned in this connection that Arrhenius (10) found 65° to be the inactivation temperature of trypsin. Considering the fact that the critical inactivation temperature of trypsin may vary from 50° to 60° or to a still higher

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value depending on the nature and extent of the inert proteins associated
with it, it is perhaps permissible to assume that the proteolytic activity of
the venoms is due to one and the same enzyme, viz., trypsin, and the differ-
ent critical inactivation temperatures observed, should be attributed to the
different kinds and quantities of proteins with which the enzyme is associated
in the different venoms.

TABLE II.

Russell's viper venom, 0.2% solution. Critical inactivation temp., 62°.

Venom heated for 1 hour at	0.01N-Alkali required after 2 hrs. digestion.		Diff. between I and II
	Venom + Substrate I	Control. II	
36°	7.45 c.c.	5.65 c.c.	1.80 c.c.
50°	7.40	5.65	1.75
60°	6.75	5.65	1.10
62°	6.55	5.65	0.90
65°	6.05	5.65	0.40
70°	5.70	5.65	0.05

Cobra venom, 0.4% solution. Critical inactivation temperature, 53°.

36°	7.10 c.c.	6.45 c.c.	0.65 c.c.
50°	6.90	6.45	0.45
55°	6.60	6.45	0.15
60°	6.50	6.45	0.05
65°	6.45	6.45	0.00

Krait venom, 0.4% solution. Critical inactivation temperature, 55°.

36°	5.70 c.c.	5.40 c.c.	0.30 c.c.
50°	5.65	5.40	0.25
55°	5.55	5.40	0.15
60°	5.40	5.40	0.00
65°	5.40	5.40	0.00

Echis Carinata venom, 0.1% solution. Critical inactivation temperature 55°.

36°	6.80 c.c.	5.30 c.c.	1.50 c.c.
50°	6.40	5.30	1.10
55°	6.10	5.30	0.80
60°	5.60	5.30	0.30
65°	5.30	5.30	0.00

Enzyme used—trypsin (E. Merck 0.2% solution). Critical inactivation temperature, 50°. Enzyme heated for 1 hour.

Venom heated for 1 hour at	0.01N-Alkali required after 2 hrs. digestion.		Diff. between I and II
	Venom + Substrate (I)	Control (II)	
30°	6.80 c.c.	5.45 c.c.	1.35 c.c.
45°	6.35	5.45	0.90
50°	6.15	5.45	0.70
55°	5.55	5.45	0.10

Enzyme preparation used—trypsin dissolved in 2% cobra venom solution previously heated to 70°. Substrate used, casein 1.7%. Critical inactivation temperature, 60°.

30°	10.65 c.c.	5.85 c.c.	4.80 c.c.
36°	10.65	5.85	4.80
50°	10.30	5.85	4.45
55°	9.65	5.85	3.80
60°	8.20	5.85	2.35
65°	6.75	5.85	0.90

ACTION OF SNAKE VENOM ON THE ACTIVITY OF TRYPSIN:

Delezenne (2) noticed that although snake venom alone could not digest egg albumen coagulated by heat, yet when it was added to inert pancreatic juice it led to the rapid digestion of the coagulated protein. On heating to 100°C for 15 minutes the solution of venom was found to have lost its power of activating the inert pancreatic juice. In our experiments we tried the effect of ordinary as well as heated solutions of venoms on the activity of trypsin. The substrates used were casein and gelatin and their concentration in the final mixture containing the enzyme was 1.7%. The concentration of the heated or the ordinary venom in all the flasks in which they were added was 0.1% and the concentration of trpsin in all the flasks in which it was added was kept constant. The final volume of the mixture in each case was 20 c.c. The extent of hydrolysis was determined by withdrawing 5 c.c. of solution at regular intervals and titrating it by Sorensen's method. The results obtained with casein only are recorded in Table III, in which h.v. stands for heated solution of venom, v for ordinary (i.e., not heated) solution of venom, T for solution of trypsin, h.v + T for mixtures of heated venom and trypsin and v + T for mixtures or ordinary venom and trypsin. The heated solutions of the venoms were prepared by heating them at 70°C for one hour. It will be noticed from the data recorded in Table III that the addition of solutions of the venoms of Naja Naja, B. Fasciatus and Echis Carinata previously heated at 70° for one hour, increases the activity of trypsin markedly. The venoms of B. Fasciatus and Echis Carinata, even when they are not heated, activate trypsin but solutions of Naja Naja venom, when it is not heated and Vipera Russellii venom whether heated or not, inhibit the activity of trypsin to an appreciable extent.

TABLE III.
Cobra venom.

pH.	Period of digestion.	h.v.	v.	T.	h.v.+T.	v.+T.	Control.
				No. of c.c. of 0.01N-alkali required.			
7.1	0 hrs.	4.45	4.45	4.45	4.45	4.45	4.40
	3 hrs.	4.45	4.70	8.80	9.70	7.40	4.40
Diff.		0.00	0.25	4.35	5.25	2.95	0.00
8.0	0 hrs.	4.40	4.40	4.40	4.40	4.40	4.40
	3 hrs.	4.40	4.70	8.45	10.15	7.15	4.40
Diff.		0.00	0.30	4.05	5.75	2.75	0.00

B. Fasciatus venom.

pH.	Period of digestion.	h.v.	v.	T.	h.v.+T.	v.+T.	Control.
7.0	0 hrs.	4.80	4.80	4.80	4.80	4.80	4.75
	3 hrs.	4.80	4.90	9.70	10.20	10.20	4.75
Diff.		0.00	0.10	4.90	5.40	5.40	0.00
8.0	0 hrs.	4.40	4.40	4.40	4.40	4.40	4.40
	3 hrs.	4.40	4.60	9.00	10.05	9.50	4.40
Diff.		0.00	0.20	4.60	5.65	5.10	0.00

Russell's viper venom.

pH.	Period of digestion.	h.v.	v.	T.	h.v.+T.	v.+T.	Control.
7.0	0 hrs.	4.95	4.95	4.95	4.95	4.95	
	3 hrs.	4.95	5.35	8.80	6.80	7.20	
Diff.		0.00	0.40	3.85	1.85	2.25	

Echis Carinata venom.

pH.	Period of digestion.	h.v.	v.	T.	h.v.+T.	v.+T.	Control.
7.0	0 hrs.	4.95	4.95	4.95	4.95	4.95	4.95
	3 hrs.	4.95	6.40	8.95	10.70	11.65	4.95
Diff.		0.00	1.45	4.00	5.75	6.70	0.00
8.0	0 hrs.	4.35	4.35	4.35	4.35	4.35	4.35
	3 hrs.	4.35	5.60	8.15	10.00	10.85	4.35
Diff.		0.00	1.25	3.80	5.65	6.50	0.00

ACTION OF SNAKE VENOM ON WITTE'S PEPTONE:

It has been recorded by Lannoy (4) that disintegration of proteins like casein and serum albumen by the action of the venoms of the cobra and the viper groups of snakes proceeds to the stage of albumose. It never leads to the formation of peptone. It is not yet known whether the venoms of Naja Naja or Vipera Russellii can hydrolyse peptone to simpler compounds. The action of the venoms of Naja Naja, B. Fasciatus, Echis Carinata and Vipera Russellii on solutions of Witte's peptone at different pH was, therefore, investigated. To each of a series of conical flasks containing 7 c.c. of 5% peptone solution adjusted to the requisite pH, were added 5 c.c. of buffer

solution of the same pH, 2 c.c. of solution of venom and 8 c.c. of physiological saline. The course of hydrolysis was followed in the same manner as described previously. Some of these results are recorded in Table IV. It has been observed that all these venoms can hydrolyse Witte's peptone and their optimum activity is in the neighbourhood of pH 8.4.

TABLE IV

B. Fasciatus venom. Incubation period—14 hours.
0.045 N KOH required to titrate 5 c. c. of solution.

pH	Venom + Substrate	Control	Difference
6.0	5.96	5.58	0.38
6.6	5.14	4.70	0.44
7.0	4.64	4.10	0.54
7.6	3.80	3.15	0.65
8.0	3.25	2.56	0.69
8.5	2.62	1.92	0.70
9.0	2.20	1.54	0.66

Vipera Russellii venom.
0.046 N KOH required in c. c. to titrate 5 c. c. of solution.

pH	Venom + Substrate	Control	Difference
6.0	5.77	5.52	0.25
6.6	4.96	4.66	0.30
7.0	4.44	4.08	0.36
7.6	3.56	3.10	0.46
8.0	3.05	2.55	0.50
8.6	2.47	1.94	0.53
9.0	1.98	1.50	0.48
9.4	1.65	1.25	0.40

DIPEPTIDASE.

The properties of purified dipeptidase, prepared from yeast and gut of animals, have been studied by Grassmann and Dyckerhoff (11) and Waldschmidt-Leitz, Balls and Waldschmidt-Grasser (12). They have found that this enzyme can hydrolyse dipeptides like glycylglycine which contains no asymmetric carbon atom and hence optically inactive and glycyl-*l*-leucine, which occurs in nature. In the course of our investigation on the enzymes in snake venoms, we have found a dipeptidase in the venoms of cobra (Naja Naja), banded krait (B. Fasciatus), Echis Carinata and Vipera Russellii. Like the gut or yeast dipeptidase, this peptidase in venom can hydrolyse glycylglycine and *l*-leucylglycine. This will be evident from the date recorded in Table V. The course of hydrolysis was followed by Sorensen's method of formol titration. The concentration of the substrates used was 0.008M and the concentration of the venoms used was 0.1 per cent, in each case. The pH of the substrate + venom solution was 7.0.

TABLE V

0.01N-NaOH required (in c.c.) for titration of 5 c.c. of the solution after 24 hours' incubation at 36°.

Cobra venom.

Substrate		Control.	Venom + substrate.	Diff.
l-Leucyl-*l*-tyrosine	...	1.52 c.c.	2.42 c.c.	0.90 c.c.
l-Leucylglycine	...	1.30	1.74	0.44
d-Leucylglycine	...	1.30	1.30	0.00
Glycylglycine	...	8.80	9.45	0.65

Russell's viper.

l-Leucyl-*l*-tyrosine	...	1.55 c.c.	2.65 c.c.	1.10 c.c.
l-Leucylglycine	...	1.25	1.60	0.35
d-Leuclyglycine	...	1.25	1.25	0.00
Glycylglycine	...	8.80	9.55	0.75

B. Fasciatus.

l-Leucyl-*l*-tyrosine	...	1.55 c.c.	2.11 c.c.	0.56 c.c.
l-Leucylglycine	...	1.20	1.85	0.65
d-Leucylglycine	...	1.25	1.25	0.00
Glycylglycine	...	8.70	9.40	0.70

Echis carinata.

l-Leucyl-*l*-tyrosine	...	1.60 c.c.	4.80 c.c.	3.20 c.c.
l-Leuoylglycine	...	1.40	2.55	1.15
d-Leucylglycine	...	1.40	1.40	0.00
Glycylglycine	...	8.75	9.55	0.80

POLYPEPTIDASE.

The properties of purified polypeptidase obtained from yeast and gut of animals have been investigated by Grassmann and Dyckerhoff (11) and Waldschmidt-Leitz and Balls (13). It has been found that this enzyme attacks the naturally occurring *laevo*-rotatory forms of the polypeptides only and not the *dextro*-rotatory forms. We have found the presence of this enzyme in the venoms of Naja Naja, Vipera Russelli, Echis Carinata and B. Fasciatus. The results are recorded in Table VI. The concentration of the solutions with respect to the venoms was 0.1 per cent. in all the cases recorded below. It will be noticed from the data in Table VI that the enzyme can hydrolyse *l*-leucylglycylgylcine and not *d*-leucylglycylglycine. This seems to indicate that the polypeptide molecule is attacked by the enzyme on the end containing the free amino group.

TABLE VI

0.01N-NaOH solution required (in c.c.) for the titration of 5 c.c. of the solution after 24 hours' incubation at 36°.

Cobra venom.

Substrate		Control.	Venom + substrate.	Diff.
l-Leucylglycylglycine	...	1.40 c.c.	5.40 c.c.	4.00 c.c.
d-Leucylglycylglycine	...	1.40	1.40	0.00

Russell's viper.

l-Leucylglycylglycine	...	1.35 c.c.	2.36 c.c.	1.01 c.c.
d-Leucylglycylglycine	...	1.35	1.35	0.00

B. Fasciatus.

l-Leucylglycylglycine	...	1.40 c.c.	4.10 c.c.	2.70 c.c.
d-Leucylglycylglycine	...	1.40	1.40	0.00

Echis carinata.

l-Leucylglycylglycine	...	1.40 c.c.	5.66 c.c.	4.26 c.c.
d-Leucylglycylglycine	...	1.40	1.40	0.00

CARBOXYPOLYPEPTIDASE.

Carboxypolypeptidase has been obtained in crystalline form by Anson (14). Bergmann, Zervas and Schleich (15) have shown that it can hydrolyse peptides of the form XCONH.CRH.COOH, where X and R stand for uni-valent radicals. We searched for this enzyme in the venoms of Naja Naja, B. Fasciatus, Echis Carinata and Vipera Russellii using chloroacetyltyrosine as substrate. It will be noticed from the data recorded in Table VII that the venoms mentioned above contain appreciable amount of carboxypoly-peptidase. In the following experiments the concentration of the different venoms in their respective solutions was 0.1 per cent, the concentration of the substrate was 0.008M, and the pH of the solutions was 7.0 in each case.

TABLE VII

Substrate Chloroacetyl-l-tyrosine.

0.01N-NaOH solution required (in c.c.) for the titration of 5 c.c. of the solution after 24 hours' incubation at 36°.

Type of venom used.		Control.	Venom + substrate.	Diff.
Naja Naja	...	2.70	3.46	0.76
Vipera Russellii	...	2.65	3.20	0.55
B. Fasciatus	...	2.65	3.20	0.55
Echis Carinata	...	2.70	5.25	2.55

CHOLINE-ESTERASE.

The existence of a choline-esterase was first suggested by Dale (16). It occurs in the blood and tissues such as heart muscle, intestinal mucosa, etc. of animals. It has been shown by Stedman and co-workers (17) and Plattner and co-workers (18) that it is specific in its action since it does not parallel the lipase content of various organs. Iyengar and co-workers (7) have recently found that Cobra (Naja Naja) venom possesses considerable cholin-esterase activity, whereas Vipera Russellii shows practically no such activity. We have also found that Naja Naja and B. Fasciatus venoms possess marked choline-esterase activity, while the venoms of Echis Carinata, Vipera Russellii and Crotalus-t-terrificus show no such activity. This will be noticed from the data recorded in Table VIII. Since acetylcholine is liberated from nerve-endings and mediates in the transmission of nerve impulse and since in cobra poisoning there occurs paralysis of the nervous system, Iyengar and co-workers (7) put forward the hypothesis that the neurotoxin of cobra venom is probably identical with choline-esterase. They meant thereby that when a cobra neurotoxin is introduced into the animal system in sufficient amount, it causes immediate destruction of the acetyl-choline liberated, and thus produces paralysis of the nervous system. We have, however, found that the purified cobra neurotoxin (M.L.D. 1/90 mg.) prepared by us does not possess choline-esterase activity. Furthermore, when solutions of crude cobra and B. Fasciatus venoms are heated at 60° or 70°, for 30 minutes they completely lose there choline-esterase activity, while their neurotoxic activity remains practically unaltered. This will be evident from the data recorded in Table IX. The concentration of the substrate solutions was 0.625 per cent. in those cases where cobra venom was used ; in the other cases it was 0.315 per cent. The pH of the solutions was adjusted to 7.0 in each case.

TABLE VIII

0.0143N-NaOH required (in c.c.) for the titration of 10 c.c. of the solution after 3 hours' incubation at 36°.

Type of venom.		Conc. of venom.	Control.	Venom + substrate.	Diff.
Naja Naja	...	0.0125%	4.05	10.90 c.c.	6.25 c.c.
B. Fasciatus	...	0.0250	4.10	15.80	11.75
Vipera Russellii	...	0.1250	4.10	4.10	0.00
Echis Carinata	...	0.0125	4.10	4.10	0.00
Crotalus-t-terrificus	...	0.0125	4.10	4.10	0.00

TABLE IX

0.0143N-NaOH required (in c.c.) for the titration of 10 c.c. of the solution after 3 hours' incubation at 36°.

Type of venom.	Venom heated at	Conc. of venom.	Control.	Venom + substrate.	Diff.	Toxicity remaining.
Naja Naja crude	60°	0.0125%	4.05 c.c.	4.05 c.c.	0.00 c.c.	95%
Do.	70°	0.0125	4.05	4.05	0.00	90
Naja Naja purified nourotoxin	36°	0.0125	4.05	4.05	0.00	100
B. Fasciatus	60°	0.025	4.05	4.10	0.05	95
Do.	70°	0.025	4.05	4.05	0.00	80

ISOLATION OF THE HÆMOLYSIN:

Kyes (10) showed that by the action of cobra venom on lecithin a powerful lysin was formed. The possibility of separation of this active substance was investigated by the authors. It has been found by Ghosh and De (20) that the protein fraction which is precipitated from a solution of cobra venom at 20 per cent. NaCl concentration contains the hæmolytic agent along with a number of enzymes, e.g., the phosphatases, the proteinase etc. The hæmolysin was separated from these enzymes by the following method. 40 Gm. of solid sodium chloride were gradually added with constant stirring to 200 c. c. of a 0.5 per cent. solution of cobra venom and the mixture maintained at 37°C for one hour. The precipitate containing the hæmolysin was separated by centrifuging and washed with 20 per cent. sodium chloride solution. It was then dissolved in 100 c. c. of water and treated with 20 c.c. of a freshly prepared 20 per cent. solution of metaphosphoric acid and the mixture left at 37°C for 30 minutes. The supernatant solution containing the hæmolysin was separated from the precipitate and treated with 5 c.c. of a 10 per cent. solution of sodium tungstate and 5 c. c. of 2/3 normal sulphuric acid and the mixture allowed to stand for 15 minutes. It was then centrifuged and the precipitate containing the hæmolysin was washed thrice with a dilute solution of sodium tungstate and sulphuric acid. It was then dissolved in a dilute solution of caustic soda and treated with barium chloride to precipitate the tungstic and phosphoric acids. The supernatant solution was withdrawn and the excess of barium was removed by precipitation with sodium sulphate. The solution was then evaporated to dryness inside a vacuum desiccator. The dry residue was dissolved in 5 c. c. of water and treated with 20 c. c. of pyridine cooled below 4°C. The precipitate formed was inactive and was removed. The supernatant solution was evaporated to dryness and the residue disolved in 10 c.c. of 0.9 per cent. sodium chloride solution and treated with 48 mg. of freshly prepared ferric hydroxide gel and shaken for one hour. The ferric hydroxide absorbs the inactive proteins in preference to the hæmolysin. The gel was

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removed by centrifuging and the clear supernatant solution was withdrawn and evaporated inside a vacuum desiccator. For the same weight this hæmolysin preparation was found to be about eleven times more active than the crude venom. This purified hæmolysin possessed strong lecithinase activity but did not contain any other enzyme.

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ON THE SIGNIFICANCE OF THE S/N RATIO IN HUMAN URINE AND ON THE DISTRIBUTION OF URINARY SULPHUR ON NON-PROTEIN DIET AND ON DIETS CONTAINING DIFFERENT AMOUNTS OF PROTEINS FROM RICE AND WHOLE WHEAT.

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It is generally assumed that all sulphur and nitrogen present in the urine are derived from protein metabolism. If we accept this assumption it is to be expected that there would be a close parallelism between the excretion of the two and further that the two would be in the same proportion as in the protein undergoing catabolism. In fact, von Wendt (1) expressed the view that no true picture of protein metabolism could be obtained unless the excretion of both nitrogen and sulphur in the urine was followed simultaneously. Studies in S:N ratio in urine have revealed many important and interesting results on protein catabolism and retention, which remained obscure by studies on nitrogen determinations alone. To account for the variations in the S:N ratio of urine in feeding experiments it has been assumed by Wendt (1), Cathcart and Green (2), Wilson (3) and a number of other investigators that the sulphur-containing moiety of the protein molecule is more labile than the sulphur-free fraction, i.e., the sulphur moiety of the protein molecule is the first to be mobilised both in the breakdown and storage of protein and that there is always a delay in the excretion of nitrogen as compared with that of sulphur.

During fasting, when all protein metabolism is endogenous, the S:N ratio, at least theoretically, would be the same as that of the protein catabolised. The S:N ratio of the muscle tissue, which during inanition is drawn upon extensively, is generally accepted as 1:14. The ratios actually noted in several carefully conducted investigations on fasting humans (4, 5, 6, 7) indicate that during fasts ranging from a few days to 31 days the average S:N quotient varies between 1:15 and 1:17. It is obvious, therefore, that during fasting the muscle protein is being metabolised almost exclusively, the slight discrepancy being accounted for by the fact that probably a slight amount of other tissue proteins with a lower sulphur content is also being catabolised.

The question arises if this fasting protein metabolism is essentially the same as or different from the so-called endogenous protein catabolism when the diet is free from protein but contains sufficient amounts of energy yielding material. Studies in the S:N ratio of urine of fasting animals and

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animals on protein-free diet with sufficient energy intake might contribute an answer to the question. Investigations on this line are very few and even among these, data obtained by different workers are very confusing and contradictory. The ratio of sulphur to nitrogen in the urine when the nitrogenous catabolism is reduced to the lowest level by the feeding of nitrogen-free nutrients only, is 1:10 to 1:12 according to Folin (8). But in Wilson's (3, 9) papers which report experiments conducted on himself on a nitrogen-free diet, this ratio was found to be in the neighbourhood of 1:14.0 to 1:16.1. These results seem to be rather curious because they lead to the idea that under such condition also body protein was actually being catabolised. An attempt is made in this paper to clear up this confusion.

Folin's (8) experiments on the composition of urine of normal human subjects on standard diets of varying nitrogen and sulphur contents but practically free of purine, creatine and creatinine, suggested the hypothesis that there are two essentially different forms of protein catabolism ; the constant or endogenous catabolism yielding chiefly creatinine and neutral sulphur and to a less extent uric acid and ethereal sulphates, and the variable catabolism yielding chiefly urea and inorganic sulphates. Folin states that the more the total catabolism is reduced, the more prominent become these representatives of the constant catabolism, and the less prominent become the two representatives of the variable catabolism.

Whilst Folin's general hypothesis has been generally accepted, doubt has been expressed as to whether these various excretory products can be exactly divided into two such groups. Whereas an extensive literature has grown around the question of the partition or the distribution of the nitrogenous constituents in the urine of human subjects on both non-protein and protein-containing diets there is very little information as regards the partition of sulphur under similar conditions. The urinary sulphur, like urinary nitrogen, appears in a variety of forms which can be grouped in three classes : as salts of sulphuric acid, and as organic combinations of sulphuric acid, in both of which the sulphur is completely oxidised, and finally as neutral or unoxidised sulphur. The latter fraction is made up of a variety of sulphur-containing substances. This investigation also deals with the determination of the sulphur constituents in urine. The proteins in the diet were derived mostly from rice or wheat, the two staple food-stuffs in India. Rice or wheat was also fed at different levels.

EXPERIMENTAL

The experiments were conducted on G.C.N., a young healthy man of eighteen weighing 49 kilograms. A number of experimental diets (nitrogen-free or exclusively vegetarian) composed of known amounts of food-stuffs, as indicated in Table I, was taken for a period of six consecutive days. Urine and fæces excreted on the last three days only were collected, the

first three days being considered as a preliminary period to avoid any effect of the previous diet. The urine was collected from the morning of the experimental period quantitatively for the twenty-four hours and preserved over toluene to which some chloroform solution of thymol was added. The total volume of urine excreted each day was noted and then made up to a suitable volume for estimation. In order to be sure that the collection of urine was complete, the amount of creatinine eliminated daily was estimated and found to be fairly constant. Fæces were collected together for periods of three days, since daily dry weight of fæces was found to vary considerably. Fæces were marked by carmine. A 5-grain capsule of carmine was ingested with the first meal of the metabolism period and again with the first meal after the period ended. Fæces were preserved with a small quantity of glacial acetic acid, dried over water-bath with addition of alcohol now and then, thoroughly powdered, weighed and preserved in the refrigerator in stoppered bottles.

ANALYTICAL METHODS.

(a) Urine:—

Total nitrogen	... Kjeldahl method.
Creatinine	... Folin's method.
Total sulphur	... Denis's modification of Benedict's method.
Inorganic sulphur and ethereal sulphur	... Folin's method.
Neutral sulphur	... By difference.

(b) Fæces:—

| Total nitrogen | ... Kjeldahl method. |
| Total sulphur | ... Material evaporated to dryness with HNO_3 and then Denis's modification of Benedict's method applied. |

TABLE I

Composition of diets.

Diet	Rice (g.)	Wheat (g.)	Vegetables. (g.)	Dal. (g.)	Sago. (g.)	Ghee. (butter fat) (g.)	Sugar. (g.)
Rice diet.							
Diet 1.	250	—	200	25	350	25	100
Diet 2.	400	—	200	25	200	25	100
Diet 3.	600	—	200	25	—	25	100
Wheat diet: 1st series.							
Diet 1.	—	150	200	25	450	25	100
Diet 2.	—	400	⌐ 200	25	200	25	100
Diet 3.	—	600	200	25	—	25	100

Diet	Rice (g.)	Wheat (g.)	Vegetables. (g.)	Dal. (g.)	Sago. (g.)	Ghee. (butter fat) (g.)	Sugar. (g.)
			Wheat diet: *2nd series.*				
Diet 1.	—	150	200	25	450	25	100
Diet 2.	—	200	200	25	400	25	100
Diet 3.	—	400	200	25	200	25	100
			Non-protein diet.				
Diet 1.	—	—	—	—	600	—	150

RESULTS

NITROGEN-FREE DIET:—

Results of experiments on non-protein diet are indicated in Table II. The average total urinary nitrogen elimination is 1.499 g. which reduces to 0.03 g. nitrogen per kg. body weight. Corresponding total fæcal nitrogen elimination is 0.946 g. The elimination of total sulphur in urine on endogenous diet is 0.1737 g. and the ratio of total sulphur to total nitrogen is 1:8.5.

The results of experiments on sulphur partition on varying protein containing diets are arranged in Tables III, IV and V and graphically represented in figures I, II and III.

RICE DIET:

Total nitrogen:—

As the amount of rice in experimental diet at first increases and then decreases from 250 g. — 400 g. — 600 g. — 600 g. — 400 g. — 250 g. (energy ingestion being kept constant) the elimination of total nitrogen in urine increases from 2.041 g. — 2.329 g. — 3.275 g. and then decreases from 3.383 g. — 2.557 g. — 2.029 g. The elimination of creatinine, however, remains almost constant throughout.

Total sulphur:—

With the increase or decrease of rice in diet the elimination of total sulphur correspondingly increases or decreases, always running practically parallel to that of total nitrogen, with the result that S:N ratio keeps almost constant, at about 1:8.0.

Inorganic sulphate:—

About 65% of the total sulphur is eliminated in this form and that it runs parallel to that of total sulphur.

Ethereal sulphate:—

Elimination of sulphur in this form is rather arbitrary, bearing no relation with that of total sulphur; the average value varies anything between 5 and 10% of total sulphur.
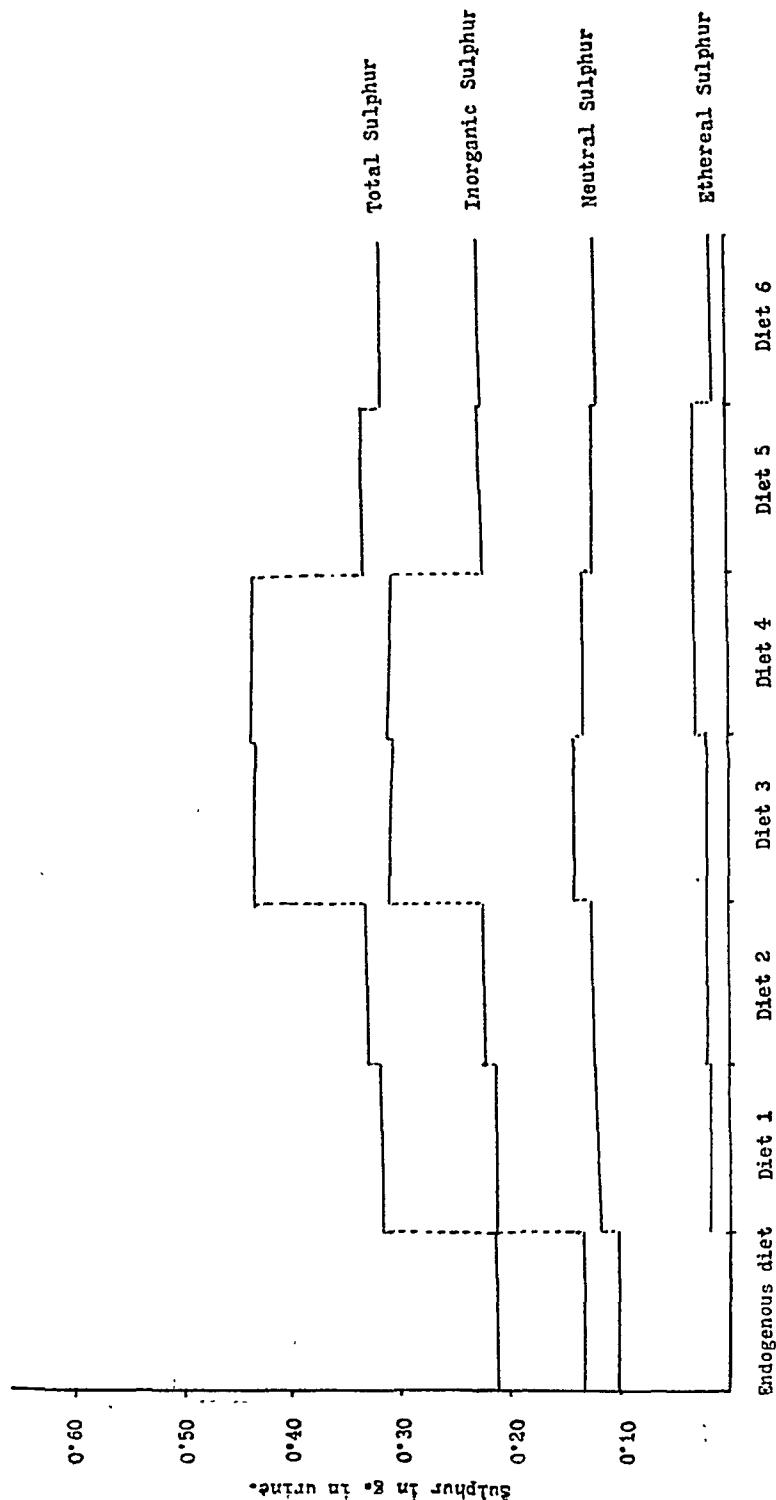
Fig. 1.

Elimination of different forms of sulphur in human urine on rice diet.
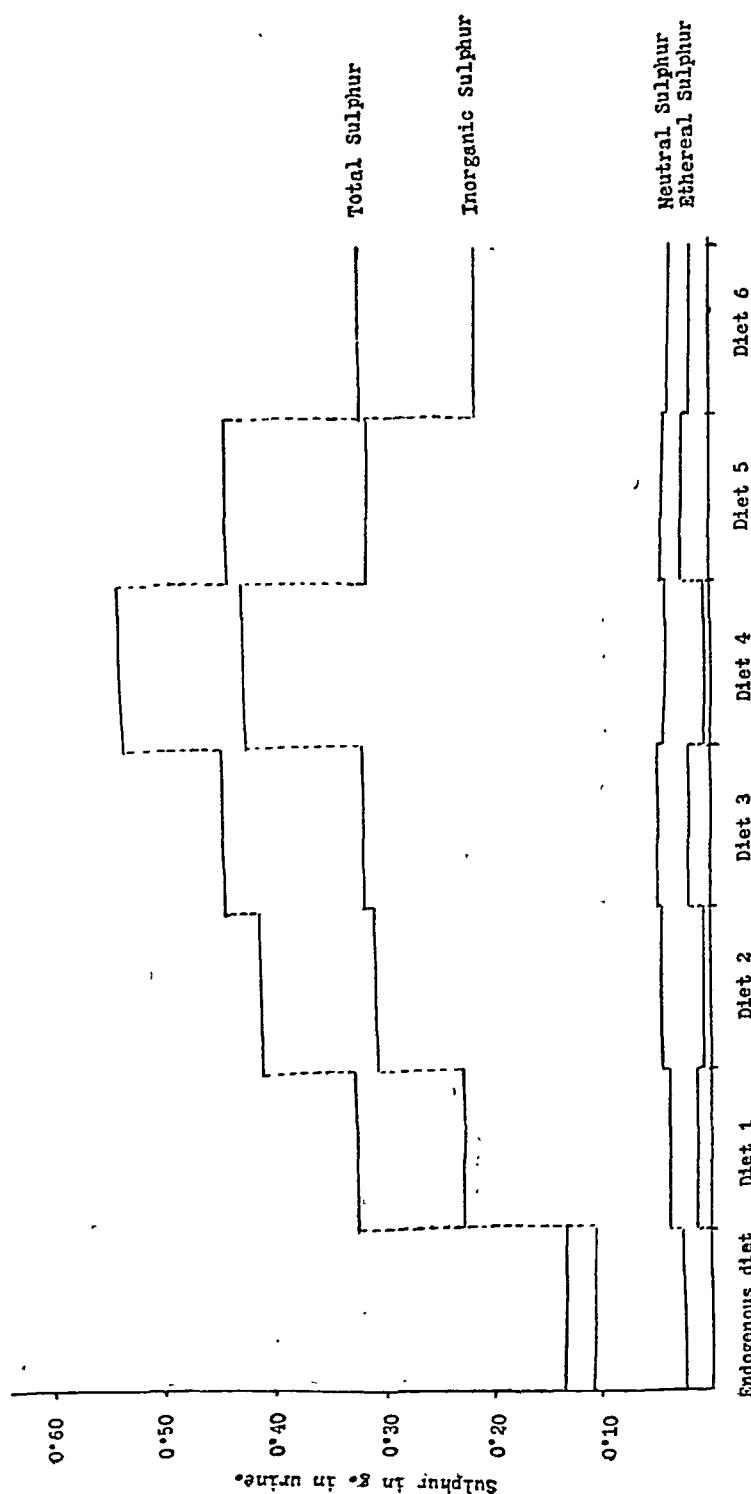
Fig. 2.

Elimination of different forms of sulphur in human urine on wheat diet (1st series).

Neutral sulphur:—

There is not quite the same constancy about this fraction as there is about creatinine. On the other hand, it increases quite consistently with the increase in protein concentration and hence in protein metabolism as measured by the increase in total sulphur. It constitutes about 30% of the total sulphur.

WHEAT DIET :

Total nitrogen:—

As in experiments with rice, the elimination of total nitrogen in urine varies with the protein content in diet. When the amount of wheat is varied as 150 g. — 400 g. — 600 g. — 600 g. — 400 g. — 150 g., nitrogen elimination follows the course, 2.626 g. — 3.328 g. — 4.961 g. — 5.357 g. — 3.984 g. — 1.892 g. The elimination of creatine is remarkably constant throughout as in experiments with rice.

Total sulphur:—

Elimination of total sulphur bears close relation to that of total nitrogen. When the protein content of the diet is increased, the sulphur content of urine rises but the rise is not so much as that of nitrogen with the result that the S:N ratio continually rises from 1:7.4 to 1:9.7.

Inorganic sulphate:—

There is an increase in the amount of inorganic sulphate excreted but the percentage of sulphur (about 70%) excreted in this form remains very nearly constant.

Ethereal sulphate:—

Elimination of ethereal sulphate is quite arbitrary.

Neutral sulphur:—

This is not so remarkably constant as creatinine, but continually increases with increasing protein content in the diet. Its average value varies between 16 and 20%.

TABLE II

S : N ratio on protein-free diet.

Day of expt.		Urine volume in c.c.	Total N. (g.)	Total S. (g.)	Inorganic S. (g.)	Ethereal S. (g.)	Neutral S. (g.)	S:N.
4th	...	1790	1.459	0.1732	0.1279 (73.86%)	nil	0.0453 (26.14%)	1:8.4
5th	...	1595	1.934	0.2166	0.1443 (68.21%)	nil	0 0723 (31.79%)	1:8.9
6th	...	1390	1.104	0.1314	0.0866 (67.42%)	nil	0 0428 (32.58%)	1:8.4
Average	...	1572	1.499	0.1737	0 1236 (71.32%)	nil	0.0535 (28.68%)	1:8.5

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TABLE III

S : N ratio and distribution of sulphur in urine on rice diet.

Diet No.	Day of expt.	Urine volume in c.c.	Total urinary N. (g.)	Creatinine N. (g.)	Total S. (g.)	Inorganic S. (g.)	Ethereal S. (g.)	Neutral S. (g.)	Total urinary S:N.
Diet I. 250 g. rice.	4th	1690	1.820	0.38	0.2768	0.1719 (62.10%)	0.0204 (7.37%)	0.0845 (30.53%)	1:6.4
	5th	2655	2.252	0.37	0.2928	0.2056 (70.23%)	0.0038 (1.30%)	0.0834 (28.47%)	1:7.9
	6th	2620	2.052	—	0.2866	0.1559 (54.39%)	0.0271 (9.48%)	0.0336 (35.13%)	1:7.2
	Average.	2322	2.041	—	0.2854	0.1778 (62.24%)	0.0171 (5.99%)	0.0872 (31.77%)	1:7.2
Diet II. 400 g. rice.	10th	2515	2.298	0.38	0.2917	0.1894 (64.71%)	0.0069 (2.96%)	0.0954 (32.33%)	1:7.9
	11th	2890	2.358	0.43	0.3146	0.1875 (59.60%)	0.0392 (12.47%)	0.0879 (27.93%)	1:7.5
	12th	2340	2.331	0.37	0.2997	0.1981 (66.10%)	0.0205 (6.84%)	0.0811 (27.42%)	1:7.8
	Average.	2582	2.329	0.39	0.3020	0.1917 (63.48%)	0.0222 (7.36%)	0.0881 (29.16%)	1:7.7
Diet III. 600 g. rice.	16th	2350	3.022	0.38	0.3875	0.2480 (64.00%)	0.0285 (7.35%)	0.1110 (28.65%)	1:7.8
	17th	2615	3.464	0.37	0.4011	0.2630 (65.58%)	0.0259 (6.55%)	0.1122 (27.87%)	1:8.6
	18th	2550	3.328	0.37	0.4163	0.2733 (65.66%)	0.0085 (2.04%)	0.1345 (32.30%)	1:8.0
	Average.	2505	3.271	0.37	0.4016	0.2614 (65.10%)	0.0220 (5.48%)	0.1192 (29.42%)	1:8.1

TABLE III (*Continued*)

Diet No.	Day of expt.	Urine volume in c.c.	Total urinary N. (g.)	Creatinine N. (g.)	Total S. (g.)	Inorganic S. (g.)	Ethereal S. (g.)	Neutral S. (g.)	Total urinary S:N.
Diet III. 600 g. rice.	22nd	2600	3.377	0.37	0.3959	0.2473 (62.30%)	0.0229 (5.78%)	0.1257 (31.92%)	1:8.5
	23rd	2700	3.418	0.38	0.4081	0.2686 (65.82%)	0.0298 (7.30%)	0.1097 (26.88%)	1:8.4
	24th	2570	3.355	0.33	0.3758	0.2430 (64.68%)	0.0296 (7.88%)	0.1032 (27.44%)	1:8.9
	Average.	2623	3.383	0.36	0.3932	0.2530 (64.34%)	0.0274 (6.97%)	0.1129 (28.69%)	1:8.6
Diet II. 400 g. rice.	28th	2760	3.026	0.40	0.3399	0.2078 (61.13%)	0.0350 (10.30%)	0.0971 (28.57%)	1:8.9
	29th	2680	1.993	0.30	0.2666	0.1650 (61.89%)	0.0191 (7.16%)	0.0825 (30.95%)	1:7.5
	30th	2640	2.653	0.37	0.3120	0.1929 (61.81%)	0.0304 (9.74%)	0.0887 (28.54%)	1:8.5
	Average.	2693	2.557	0.36	0.3061	0.1886 (61.63%)	0.0282 (9.21%)	0.0894 (29.16%)	1:8.3
Diet I. 250 g. rice.	34th	2470	2.136	0.36	0.2512	0.1643 (65.40%)	0.0027 (1.17%)	0.0842 (33.43%)	1:8.5
	35th	2465	1.775	0.34	0.2533	0.1608 (63.49%)	0.0167 (6.59%)	0.0758 (29.92%)	1:7.0
	36th	2990	2.176	0.38	0.3008	0.2284 (75.95%)	0.0033 (1.10%)	0.0691 (22.95%)	1:7.2
	Average.	2642	2.029	0.36	0.2684	0.1880 (70.04%)	0.0076 (2.83%)	0.0764 (27.13%)	1:7.5

TABLE IV

S : N Ratio and distribution of sulphur in urine on wheat diets (first series).

Diet No.	Day of expt.	Urine volume in c.c.	Total urinary N. g.	Creatinine N. (g.)	Total S.	Inorganic S. (g.)	Ethereal S. (g.)	Neutral S. (g.)	Total urinary S:N.
Diet I. 150 g. wheat.	4th	1190	2.967	0.37	0.3512	0.2748 (78.27%)	0.0105 (3.00%)	0.0669 (18.73%)	1:8.4
	5th	1580	2.697	0.42	0.2344	0.2344 (50.31%)	0.0694 (18.79%)	0.0662 (30.90%)	1:7.3
	6th	1190	2.213	0.36	0.3456	0.2568 (74.30%)	0.0132 (3.82%)	0.0755 (21.88%)	1:6.4
	Average	1320	2.626	0.38	0.3556	0.2553 (71.80%)	0.0310 (8.73%)	0.0692 (19.47%)	1:7.4
Diet II. 400 g. wheat.	10th	1000	3.022	0.37	0.3993	0.2947 (73.98%)	0.0159 (3.99%)	0.0887 (22.03%)	1.7.6
	11th	1260	4.040	0.40	0.4723	0.3508 (74.29%)	0.0299 (6.33%)	0.0916 (19.38%)	1:8.6
	12th	980	2.922	0.33	0.3931	0.2999 (76.30%)	0.0017 (0.43%)	0.0915 (23.27%)	1:7.4
	Average.	1080	3.328	0.36	0.4215	0.3151 (73.05%)	0.0158 (3.75%)	0.0906 (23.20%)	1:7.8
Diet III. 600 g. wheat.	16th	1140	4.759	0.26	0.3773	0.2743 (72.68%)	0.0053 (1.80%)	0.0978 (25.55%)	1:
	17th	760	4.590	0.33	0.4699	0.2968 (63.17%)	0.0773 (16.45%)	0.0958 (19.38%)	1:9.8
	18th	770	5.534	0.35	0.5735	0.4378 (76.33%)	0.0397 (6.92%)	0.0960 (16.75%)	1:9.6
	Average.	890	4.961	0.32	0.4735	0.3363 (71.01%)	0.0408 (8.62%)	0.0965 (20.37%)	1:9.7

TABLE IV (Continued)

Diet No.	Day of expt.	Urine volume in c.c.	Total urinary N. (g.)	Creatinine N. (g.)	Total S. (g.)	Inorganic S. (g.)	Ethereal S. (g.)	Neutral S. (g.)	Total urinary S:N.
Diet III. 600 g. wheat.	2nd	1460	5.403	0.40	0.5458	0.4415 (80.89%)	0.0119 (2.18%)	0.0924 (16.93%)	1:9.9
	23rd	940	5.500	0.38	0.5735	0.4591 (80.04%)	0.0212 (3.70%)	0.0932 (16.26%)	1:9.5
	24th	1050	5.168	0.34	0.5180	0.4129 (79.71%)	0.0216 (4.17%)	0.0835 (16.12%)	1:10.0
	Average.	1150	5.357	0.37	0.5457	0.4378 (80.06%)	0.0182 (3.33%)	0.0897 (16.61%)	1:9.8
Diet II. 400 g. wheat.	28th	1070	4.626	0.37	0.4820	0.3190 (66.19%)	0.0773 (16.04%)	0.0857 (17.77%)	1:9.6
	29th	1460	3.898	0.37	0.4591	0.3395 (73.84%)	0.0329 (7.17%)	0.0867 (18.98%)	1:8.5
	30th	940	3.427	0.35	0.4269	0.2796 (65.49%)	0.0576 (13.50%)	0.0927 (21.01%)	1:8.0
	Average.	1160	3.984	0.36	0.4560	0.3127 (68.58%)	0.0559 (12.26%)	0.0884 (19.16%)	1:8.7
Diet I 150 g. wheat.	34th	1340	1.792	0.28	0.2674	0.1445 (54.04%)	0.0574 (21.51%)	0.0655 (24.45%)	1:6.7
	35th	1150	1.882	0.31	0.2883	0.2010 (69.71%)	0.0139 (4.82%)	0.0734 (25.47%)	1:6.5
	36th	1690	2.271	0.33	0.4050	0.2973 (73.40%)	0.0473 (11.68%)	0.0604 (14.92%)	1:5.6
	Average.	1393	1.982	0.31	0.3202	0.2143 (66.92%)	0.0395 (12.34%)	0.0698 (20.74%)	1:6.2

TABLE V

S : N Ratio and distribution of sulphur in urine on wheat diets (second series).

Diet No.	Day of expt.	Urine volume in c.c.	Total urinary N. (g.)	Creatinine N. (g.)	Total S. (g.)	Inorganic S. (g.)	Ethereal S. (g.)	Neutral S. (g.)	Total urinary S : N.
Diet I. 150 g. wheat.	4th	1400	2.140	0.31	0.2286	0.1509 (66.01%)	0.0095 (4.16%)	0.0682 (29.83%)	1:9.3
	5th	1710	2.614	0.34	0.2999	0.2114 (70.49%)	0.0170 (5.54%)	0.0715 (23.97%)	1:8.7
	6th	2990	2.786	0.38	0.2613	0.1808 (69.20%)	0.0131 (5.01%)	0.0674 (25.79%)	1:10.7
	Average.	2033	2.513	0.34	0.2633	0.1810 (68.74%)	0.0132 (5.01%)	0.0690 (26.25%)	1:9.5
Diet II. 200 g. wheat.	10th	1360	2.738	0.38	0.2893	0.1920 (66.36%)	0.0112 (3.87%)	0.0861 (29.77%)	1:9.5
	11th	1070	2.986	0.41	0.3277	0.2287 (69.81%)	0.0105 (3.20%)	0.0885 (23.99%)	19:.1
	12th	1770	3.033	0.38	0.3211	0.2102 (65.46%)	0.0408 (12.71%)	0.0701 (21.83%)	1:9.4
	Average.	1400	2.919	0.39	0.3127	0.2103 (67.23%)	0.0208 (6.65%)	0.0816 (25.11%)	1:9.3
Diet III. 400 g. wheat.	16th	1740	4.699	0.30	0.4142	0.2726 (65.82%)	0.0516 (13.78%)	0.0900 (20.40%)	1:11.6
	17th	1650	4.033	0.35	0.3519	0.2041 (57.99%)	0.0571 (16.23%)	0.0907 (25.78%)	1:11.5
	18th	2515	4.943	0.34	0.4727	0.2948 (62.37%)	0.0498 (10.54%)	0.1243 (27.09%)	1:10.4
	Average.	1968	4.558	0.33	0.4129	0.2572 (62.29%)	0.0528 (12.78%)	0.1050 (24.93%)	1:11.0

DISCUSSION

Sulphur : Nitrogen ratios:—

In the present case the ratio (S:N) in urine of the subject on adequate energy-containing, protein-free diet is found to be 1:8.5, a value nearer to that given by Folin (8) (1:10 to 1:12) than to that found by Wilson (3, 9) (1:14.0 to 1:16.1). The ratio tallies admirably with that 1:8.3) given by Smith (10). Smith's experimental subject was kept on protein-free diet for about 31 days. Total urinary nitrogen and sulphur were estimated daily and it was observed that S:N ratio consistently decreased from 1:14.2 to about 1:7. On the day on which the nitrogen elimination was the lowest the S:N ratio was found to be 1:8.3.

Sufficient data have been recorded in the present paper to prove beyond doubt that the ratio of S:N in urine of human subjects when fed with adequate amounts of protein-free diet is 1:8.5. During fasting, however, as reported by various investigators the S:N ratio is 1:14. This means, therefore, that the protein catabolism and the endogenous catabolism are two distinct phenomena.

Before explaining the above phenomena let us discuss some allied cases. In explaining the constant concentration of amino-acid in tissues as influenced by fasting or protein-feeding, Van Slyke and Meyer (10) suggested two possibilities as to the origin and function of the free amino-acids of the tissues: first, that the amino-acids might serve as a reserve energy supply or as a reserve of tissue building material, and secondly, that their presence in the tissues might be dependent simply upon the fact that they are intermediate steps in the construction and breakdown of tissue proteins, originating either from absorbed food products or autolysing tissue protein. From the fact that starvation does not decrease the concentration of amino-acids in the tissues, these investigators concluded that the second possibility was correct.

However, from the fact that the concentration of amino-acids in the tissues is not diminished on continued feeding with a non-protein ration, which may be presumed to reduce the protein catabolism to a minimum while affording no building materials for an anabolism of protein, it does not seem probable that the presence of amino-acids in tissues is merely incidental to protein metabolism. Further it seems to be true that the intermediary products of metabolism do not normally accumulate to any considerable extent in the tissues, as is shown by the absence of demonstrable amounts of the hydroxy and keto-acids that must be produced in the breakdown of glucose, fatty acids and amino-acids ; nor do even the end products of metabolism accumulate normally to any great extent. It is fair to presume, therefore, that the free amino-acids of the tissues are not functioning as reserve material nor are they present merely as intermediary steps in the synthesis and disintegration of protein, but *they are performing some distinct and important functions in the life of the tissues,*

since such an effective mechanism exists for maintaining their concentration constant. Their possible function in the maintenance, of osmotic pressure within the, cell (II) and particularly within the nucleus, which seems to be free from inorganic electrolytes, may also be cited. .
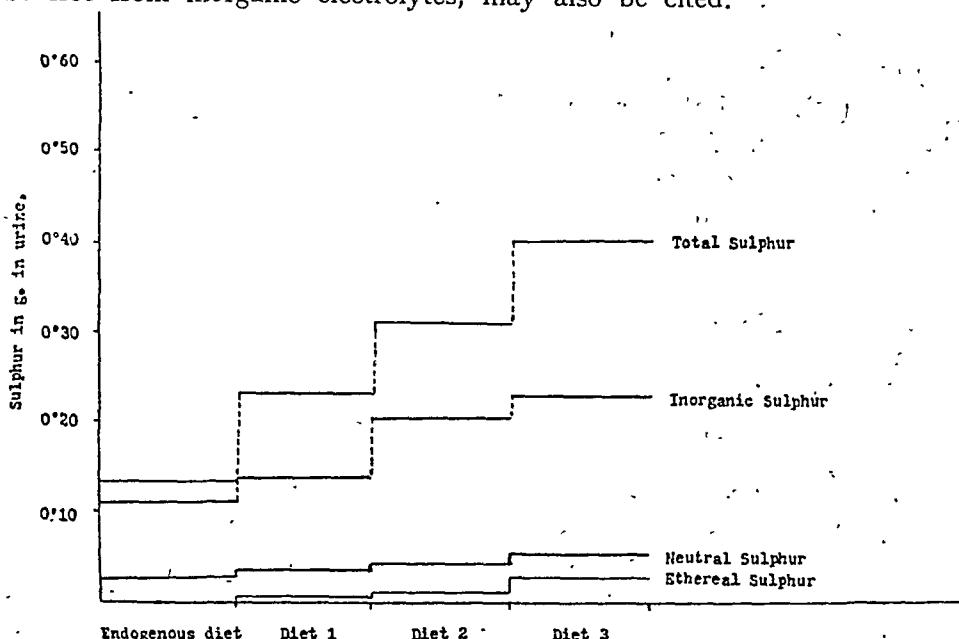

Fig. 3.

Elimination of different forms of sulphur in human urine on wheat diet
(2nd series).

The data obtained in the investigation of Mitchell, Nevens and Kendall (12) also indicate that the concentration of the total non-protein nitrogen and sulphur and of the amino-nitrogen of the tissues are not definitely affected by the type or intensity of the protein metabolism. They assume that probably most of the substances contributing to the non-protein fraction of tissue nitrogen and sulphur are performing some specific ·functions in metabolism. The universal presence of creatine in vertebrate muscle, the fact that it is not an obligate stage in the catabolism of arginine or any other known amino-acid, its· differential occurrence in different tissues, and its extremely small concentration in the blood as compared with its concentration in the tissues, constitute strong presumptive evidence that it is serving a function in tissue metabolism. Similarly with carnosine. This substance is a dipeptide containing amino-acid, β-alanine, not thus far found in proteins, and is extremely resistant to tissue proteases (13), both facts arguing a purposive synthesis in muscle cells. In the same category may be placed the autoxidisable tripeptide of Hopkins (14) namely glutathione. This compound performs important functions in the chemical dynamics of the cell.

The relation of the endogenous catabolism to these compounds—and there are probably many others that may be so classed—is an interesting

subject of speculation concerning which little definite information is available. Being soluble and diffusible, and as a class, less stable to oxidation than the cell proteins themselves, one might suppose that their loss from the tissues by purely physical means, or their destruction by catabolism agents, would be more constant, certainly more inevitable, than the loss of the proteins in the cell. All these facts and arguments prove almost conclusively the difference between the two forms of catabolism namely, the endogenous catabolism and the protein catabolism. From the above facts it might be concluded that the endogenous metabolism is the metabolism of the non-protein nitrogenous and sulphur constituents of the tissues, whereas the protein catabolism is the disintegration or autolysis of the tissue proteins.

Studies in S:N ratio support the above conclusion. In fasting, when the body protein is being disintegrated S:N of the urine should be theoretically the same as that in tissue protein, which is actually found in experiments, the two ratios coinciding at 1:14. Similarly, if the endogenous catabolism would be the same as protein disintegration, the S:N ratio in urine would be 1:14 as above. But in contrast to this, the ratio is 1:8.5 in the latter case. So we prove definitely that this endogenous metabolism is different from fasting metabolism.

We shall now discuss what the nature of the endogenous metabolism is. It has been definitely shown by Mitchell, Nevens and Kendall (12) that whereas the ratio of total sulphur to total nitrogen in the bodies of rats, previously fed on protein and non-protein diets, varies between 1:11.4 and 1:11.9, the ratio of sulphur to nitrogen in the corresponding protein-free tissue extracts of the same rats varies between 1:6.3 and 1:8.4. In the present investigation the ratio of S:N in urine on endogenous diet is 1:8.5. This proves indirectly, therefore, that under endogenous condition, the non-protein constituents of the tissues are being metabolised.

Sulphur partition:—

When the amount of protein in the various diets was increased by the addition of rice or wheat, the total nitrogen and total sulphur intake and also elimination in urine correspondingly increased. Elimination of inorganic sulphur also increased quite consistently with the increase of total sulphur elimination, forming about 65—75% of the latter, while neutral sulphur elimination was fairly constant throughout. The elimination of ethereal sulphate, however, was very arbitrary forming 2—12% of the total sulphur on protein-containing diets. Ethereal sulphates were wholly absent on non-protein diet. From these observations, it follows, that since inorganic sulphate forms 70% of the total sulphate in urine and increased with increasing protein intake, it is the chief representative of the exogenous sulphur metabolism, while constant excretion of the neutral sulphur shows that it represents products of some processes of endogenous sulphur metabolism that is little influenced by outside factors such as diets (8a).

The arbitrary nature of the excretion of ethereal sulphates on protein

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diets seems to show that probably it is not a true representative of either exogenous or endogenous protein metabolism but that it is purely a product formed in the process of detoxication of phenolic substances. In the process of digestion in the alimentary tract, and especially in the lower parts of the intestine where the bacterial flora is profuse, phenol derivatives of various types, some of them toxic in nature, are produced by the bacterial decomposition of proteins. These are absorbed into the blood stream, to be excreted later in the urine. A certain portion of them, however, upon reaching the liver becomes combined with sulphuric acid and by this process of conjugation is rendered less poisonous, and another portion may conjugate with cystine and excreted as marcapturic acid (15). On protein-free diet, since there is no chance of the production of toxic phenolic substances by bacterial decomposition of proteins, there should not be any formation of ethereal sulphate and this was actually found to be the case. Again, since small but similar amounts of ethereal sulphates were formed both on rice and wheat diets, it must be assumed that rice diet is not more susceptible to intestinal putrefaction than wheat diet, thus confirming the observation of Wilson (16).

SUMMARY

(1) The value of S:N ratio (1:8.5) on protein-free diet indicates that on such a diet the endogenous nitrogen metabolism represents the metabolism of non-protein nitrogenous constituents of the tissues, whereas the fasting metabolism (S:N=1:14) is the result of disintegration of body proteins.

(2) Total N, creatinine N, total S, inorganic S, and ethereal S, in the urine of a subject living on diets containing different amounts of rice and wheat have been determined.

(3) Similar amounts of ethereal sulphates were found to be excreted in urine on both rice and wheat diets, while it was absent on non-protein diet. This shows that rice and wheat diets are almost equally susceptible to bacterial putrefaction, while non-protein diet is not.

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SOME OBSERVATIONS ON THE MUSTARD OIL-ARGEMONE OIL THEORY OF EPIDEMIC DROPSY

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Epidemic dropsy made its appearance in Calcutta in 1877 (1) for the first time according to available records and since then various theories have been put forward from time to time but none of them has, so far, been able to explain very satisfactorily the nature of factors responsible for the outbreak of this disease. Thus, it has baffled the attempts of scientists for the last 62 years and is even now a mystery.

Recently the attention of scientific workers has been turned towards the old mustard oil theory, revived by Lal *et al* (2). From a fairly well-conducted survey and subsequent feeding experiments on human volunteers Lal and his co-workers have been able to show that it is the impure mustard oil which is responsible for the outbreak of this disease. It has been suggested further that the toxic substance (3) in the incriminated mustard oil is a definite chemical substance and is generally absent in pure mustard oil. This toxin in potent mustard oil may come from some other seeds which, according to these authors, are mixed with mustard seeds either wilfully as adulterant or become mixed up inadvertently, if the plants happen to grow in mustard fields as weeds.

Following up the observations of Sarkar (4) and Kamath (5) that oil from *Argemone mexicana* seeds, when taken by men accidentally, was found to give rise to symptoms of epidemic dropsy in these human subjects and from the inspection of stocks of mustard seeds in Calcutta market, where a slight admixture of argemone seeds could be noted, Lal and co-workers (6) assumed that oil from seeds of *Argemone mexicana* might be a factor responsible for the outbreak of epidemic dropsy. This belief was further strengthened by the fact that both potent (incriminated) mustard oil and mustard oil containing argemone oil respond similarly to the differential chemical and physical tests developed by Lal *et al* (7).

The theory has received very strong support in experiments of Chopra *et al* (8), who have shown that human subjects, when fed with a sample of mustard oil containing argemone oil mixed *in proportion of 2 to 10 per cent with pure mustard oil,* develop signs and symptoms of epidemic dropsy. From the data given, it appears that two ounces of this mixed oil were given daily to each of five subjects under experiment. The administration of mixed oil was stopped after eight days in one case and in the remaining four after fourteen days. All these subjects developed symptoms of epidemic dropsy after varying periods of time. From the statement, *2 to 10%,* it is not possible to know definitely the amount of argemone oil actually

given but one can safely take 10% as the concentration of this oil (in the mixed oil) to calculate the maximum amount of it required by human subjects to develop the characteristic symptoms of epidemic dropsy.

This evaluation has become necessary in view of the recent work of Pasricha *et al* (9) who have suggested that the amount of argemone oil necessary to bring about symptoms of epidemic dropsy in man is approximately 0.88 c.c., per 100 g. of body weight. Taking the sp. g. of the oil to be 0.9331 (4) this requirement comes to 3.725 g. of *argemone oil alone* per pound of body weight. In Table I a comparison between observed amounts of argemone oil (assuming a 10% concentration in the mixed oil) necessary in the above experiments of Chopra *et al* to produce epidemic dropsy symptoms and required amounts calculated according to Pasricha's standard has been made.

TABLE I

Showing a comparative study, on the same body weight basis, of observed and calculated requirements of argemone oil to produce symptoms of epidemic dropsy in human subjects.

				Amount of argemone oil necessary	
No.	Body wt. (in lbs.)	No. of days mixed oil given.	Total amount of oil given (in ounces)	Observed by Chopra *et al* —assuming a 10% concentration of argemone oil (in ounces)	Calculated after Pasricha (in ounces)
I	II	III	IV	V	VI
1.	89.0	14.0	28.0	2.80	11.69
2.	91.0	14.0	28.0	2.80	11.95
3.	125.0	14.0	28.0	2.80	16.43
4.	108.0	14.0	28.0	2.80	14.19
5.	113.0	8.0	16.0	1.60	14.85
Averages	105.2	...	25.60	2.56	13.82

If figures in columns V and VI are now compared it will be found that there is a considerable difference between them. The observed requirement of argemone oil (column V) varies from 1.6 ounces to 2.8 ounces whereas the required amount calculated after Pasricha (column VI) is much higher and varies from 11.69 ounces to 16.43 ounces—the average being 2.56 and 13.82 respectively. The average difference between observed and calculated figures is very great—the calculated value is about five and half times higher than the observed value. This difference is so wide that variation due to experimental error cannot possibly be a factor. It is necessary, therefore, that some suitable suggestions should come from authors concerned to explain this observed discrepancy.

If Pasricha's figure for human requirement be correct then other anomalous results, in experiments of Lal *et al* and Chopra *et al* on human subjects, will have to be faced also.   In Table II, a comparison has been made between observed amounts of potent mustard oil necessary to produce symptoms of epidemic dropsy in human subjects and corresponding required amounts, calculated after Pasricha, of artificial toxic oils (mustard oil containing 6% and 10% argemone oil).

<div align="center">

TABLE II

Showing a comparative study, on the same body weight basis, of observed requirements of potent mustard oil and requirements of pure argemone oil as well as artificial toxic mustard oils (mustard oil containing 6% and 10% argemone oil) calculated after Pasricha for producing symptoms of epidemic dropsy in human subjects.

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No.	Reference	No. of subject with body wt. (in lbs).	Observed requirement of potent mustard oil (in ounces)	Requirements—calculated after Pasricha		
				Pure argemone oil (in ounces)	Mustard oil containing 6% argemone oil (in ounces)	Mustard oil containing 10% argemone oil (in ounces)
I	II	III	IV	V	VI	VII
	Lal and Roy, I.J.M.R.*					
1.	Vol. 25, p. 250	A ; 108.0	17.50	14.19	236.40	141.90
2.	,, ,,	B ; 98.0	17.50	12.87	214.50	128.70
3.	,, p. 251	D ; 92.0	17.50	12.09	201.40	120.90
4.	,, p. 254	A' ; 84.0	37.00	11.04	184.00	110.40
5.	,, p. 255	B' ; 129.0	42.50	16.94	282.40	169.40
6.	,, ,,	C' ; 105.0	40.50	13.79	229.90	137.90
7.	,, p. 256	D' ; 91.0	24.00	11.95	199.20	119.50
8.	,, ,,	E' ; 120.0	29.50	15.77	262.70	157.70
9.	,, p. 257	F' ; 124.0	29.50	16.29	271.40	162.90
	I.J.M.R.,					
10.	Vol. 27, p. 204	1 ; 115.0	29.50	15.11	251.80	151.10
11.	,, p. 205	4 ; 110.0	22.70	14.45	240.80	144.50
12.	,, p. 206	5 ; 126.0	14.20	16.55	275.80	165.50
13.	,, ,,	6 ; 112.0	20.00	14.71	245.20	147.10
	Chopra et al, I.J.M.R.					
14.	Vol. 74, p. 194	1 ; 113.0	42.0	14.85	247.40	148.50
15.	,, ,,	3 ; 101.0	42.0	13.26	221.10	132.60
	Averages	108.5	28.4	14.3	237.6	142.6

*Indian Journal of Medical Research.

An explanation is necessary as to why two different concentrations of argemone oil (6% and 10%) have been used for the calculation of required amounts of artificial toxic oil in Table II. According to Lal *et al* (10) 'the nitric acid test is the more sensitive of the two chemical tests' so far developed. If mustard oil containing 10% argemone oil is tested side by side with a proved potent mustard oil in exactly the same way it will be found that there is distinct difference in the shade of colour, in the acid layer in the two cases and the intensity of coloration is much stronger in the case of mustard oil containing 10% argemone oil. Lal *et al* (11) have also shown that mustard oil containing 6% argemone oil 'gave the same re-actions as Rangpur oil'—the most potent oil recorded. Under these circum-stances, one can safely assume that even in the most potent oils the con-centration of argemone oil is not likely to be greater than 6%. However, to be on the safe side, calculation on 10% basis has also been made.

A study of figures in Table II will show clearly the anomalous nature of observed and calculated requirements of different oils. The require-ment of potent mustard oil (column IV) varies from 14.2 ounces to 42.5 ounces whereas the calculated requirement of *argemone oil alone* (column V) ranges from 11.04 ounces to 16.94 ounces—the averages being 28.4 and 14.3 respectively. If these requirement figures of argemone oil are con-verted into corresponding amounts of artificial toxic oils on 6% and 10% argemone basis it will be found that at 6% basis (column VI) the amount of oil required varies from 184.0 ounces to 282.4 ounces and at 10% basis (column VII), from 110.4 to 169.4 ounces—the average being 237.6 and 142.6 respectively. If average requirement figures are now compared it will be seen that the figure of 6% group is roughly eight and half times and of 10% group about five times that of potent mustard oil group.

It appears that incriminated mustard oil collected from the affected localities is very highly toxic. The amount of this oil necessary to produce symptoms of epidemic dropsy in human volunteers is extremely small in comparison with the huge requirement of artificial toxic mustard oil containing even 10% argemone oil. Further, the mean requirement of *argemone oil alone* is about half that of the potent mustard oil, which means that argemone oil diluted with an equal amount of pure mustard oil will resemble the toxic mustard oil of average potency. If this be true, then we arrive at the conclusion that in toxic mustard oils the con-centration of argemone oil should be near about 50%—a figure very difficult to believe and to accept in face of evidences already presented. If these abnormal results are not explained satisfactorily, they are likely to throw considerable doubt on the validity of argemone—con-taminated Mustard Oil Theory and will lead to the idea that argemone oil may not be the real factor. In that case it will be necessary to search for this supposed toxic substance elsewhere and if possible in the *mustard oil itself*.

The present author takes this opportunity to state that recently he has been able to obtain a sample of mustard oil in the laboratory by a process excluding the possibility of the presence of *Argemone mexicana* seeds, resembling in physical and chemical properties the potent mustard oil obtained from the affected areas. This oil gives markedly positive nitric acid and cupric acetate tests. It is fluorescent and exhibits a broad absorption band between the regions 2912 A.U. and 2667 A.U., the maximum being near about 2747 A.U. (Lal observed the absorption band in potent mustard oils between 2900 A.U. and 2600 A.U., the maxima being near about 2750 A.U.).

Further work is in progress and results obtained together with details of preparation of the particular sample of mustard oil will be given in a later publication.

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THE ·EFFECT OF THE ADMINISTRATION OF TOXIC SUBSTANCES ON ASCORBIC ACID METABOLISM

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In previous communications (1-2) it has been observed that during the period of intoxication caused by the injection of sublethal doses of diphtheria or tetanus toxin, a greater amount of ascorbic acid is excreted in the urine in a combined state. As considerable amounts of ascorbic acid are excreted normally in the urine in a combined state (3-4) it would appear that conjugation of ascorbic acid with toxic products of catabolism may be a normal biochemical process and this process for the removal of toxic substances may have added significance when infection from outside takes place. Ascorbic acid may thus have a normal rôle in the defence mechanism of the body. The above view is further supported by the observations of the increased elimination of combined ascorbic acid in the urine of patients suffering from acute pulmonary tuberculosis (5).

Conjugation with glucuronic acid is known to be a part of the normal detoxication mechanism. It was of interest to investigate how far ascorbic acid can also play a rôle in the mechanism of detoxication. The ability of ascorbic acid to detoxicate different types of poisons when introduced into the animal body forms therefore the subject of the present investigation. Experiments have been carried out to investigate whether increased elimination of combined ascorbic acid occurs in cases of cell-injury produced by the injection of histamine hydrochloride. Investigations on the effect of feeding widely varying substances which cause injury to the liver and other tissues, like camphor, chloral hydrate, phenol, phosphorus, copper and fluoride to guinea-pigs, have been carried out with a view to find what types of poisons are capable of detoxication by ascorbic acid. The effect of cyanide injection has also been studied.

EXPERIMENTAL.

Healthy, male guinea-pigs weighing between 250 and 300 g. were fed on a normal diet of green grass and germinated gram. During the experimental period each guinea-pig was kept in a separate metabolism cage. The urine excreted by each animal during 24 hours was collected over glacial acetic acid and was estimated for ascorbic acid (both free and combined) consecutively for 3 days before and after administration of the toxic material. The method of estimation was that described previously by Ghosh (1).

The ascorbic acid content of the adrenal, liver and kidney tissues of the guinea-pigs was also determined and compared with that of the corresponding tissues of normal animals. This observation was expected to

give some information regarding the organs which suffer maximum depletion.

The effect of the injection of histamine :

Histamine or histamine-like substances are supposed to be produced within the cell as a result of injury caused by bacterial toxins, anaphylactic shocks, antigen-antibody reaction, etc., (6-7). Hochwald (8) has observed that intravenous injection of ascorbic acid can inhibit anaphylactic shocks and also histamine shocks in guinea-pigs and he concluded that this inhibitory behaviour of ascorbic acid was due to its reducing property. Unger, Parrot and Levillian (9) have also shown that tissues of guinea-pigs which have been sensitised to horse serum, liberate histamine-like substances when placed in contact with dilute horse serum. If, however, the tissues are first soaked in dilute ascorbic acid solution, the reaction is inhibited. This inhibitory behaviour may perhaps be due to the conjugation of ascorbic acid with histamine or histamine-like substances. Therefore, the effect of the injection of histamine on the urinary excretion of ascorbic acid (both free and combined) was studied. It was found that the subcutaneous injection of 1.6 mg. of histamine dihydrochloride was sufficient to kill the animal within ½—1 hour. 0.8 Mg. of histamine injection can, however, be tolerated by the animal. The urine was collected as described before and the ascorbic acid content of the urine was likewise estimated, the same animal serving as the subject of experiment before and after histamine injection. The results are given in Table I, which show, on an average, a small decrease in free ascorbic acid and a small increase in combined ascorbic acid.

TABLE I

Mg. of ascorbic acid excreted per animal during 24 hours before and after histamine injection.

No. of Expt.	No. of days	Free ascorbic acid		Combined ascorbic acid in terms of free ascorbic acid	
		Before	After	Before	After
I	1	0.306	0.200	0.381	0.371
	2	0.244	0.112	0.346	0.346
	3	0.249	0.136	0.325	0.360
	Mean	0.266	0.149	0.350	0.362
II	1	0.250	0.132	0.325	0.400
	2	0.213	0.191	0.464	0.520
	3	0.229	0.153	0.346	0.400
	Mean	0.230	0.158	0.371	0.444

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No. of Expt.	No. of days	Free ascorbic acid		Combined ascorbic acid in terms of free ascorbic acid	
		Before	After	Before	After
III	1	0.118	0.131	0.333	0.530
	2	0.181	0.188	0.288	0.433
	3	0.204	0.153	0.400	0.381
	Mean	0.168	0.157	0.340	0.448
IV	1	0.267	0.159	0.217	0.216
	2	0.238	0.225	0.239	0.290
	3	0.234	0.200	0.235	0.285
	Mean	0.246	0.191	0.230	0.263

The effect of feeding phenol :

Phenol seems to be detoxicated by ascorbic acid when placed in contact with the latter. It has been observed that the convulsion-producing capacity of phenol in the rat could be mitigated by previous contact of the phenol with ascorbic acid. This effect of ascorbic acid led Leibowitz and Guggenheim (10-11) to suppose that phenol enters into combination with ascorbic acid. In our experiments 5 mg. of phenol dissolved in water (0.25 c. c.) were fed daily to guinea-pigs and the ascorbic acid content of urine was determined. It will be observed from Table II that the ingestion of phenol causes an increased elimination of combined ascorbic acid with a corresponding decrease in free ascorbic acid in the urine.

TABLE II

Mg. of ascorbic acid excreted per animal per day before and after phenol administration.

No. of Expt.	No. of days	Free ascorbic acid		Combined ascorbic acid	
		Before	After	Before	After
I	1	0.375	0.173	0.000	0.359
	2	0.371	0.074	0.123	0.246
	3	0.295	0.188	0.163	0.331
	Mean	0.347	0.145	0.095	0.312
II	1	0.216	0.079	0.104	0.542
	2	0.200	0.079	0.150	0.421
	3	0.291	0.100	0.105	0.366
	Mean	0.235	0.086	0.120	0.443
III	1	0.233	0.050	0.179	0.419
	2	0.183	0.107	0.235	0.359
	3	0.258	0.100	0.236	0.369
	Mean	0.231	0.085	0.227	0.382

No. of Expt.	No. of days	Free ascorbic acid		Combined ascorbic acid	
		Before	After	Before	After
IV	1	0.171	0.146	0.091	0.195
	2	0.170	0.175	0.145	0.144
	3	0.233	0.149	0.106	0.151
	Mean	0.191	0.156	0.114	0.163
V	1	0.154	0.112	0.126	0.170
	2	0.111	0.109	0.143	0.180
	3	0.146	0.117	0.152	0.116
	Mean	0.137	0.112	0.140	0.155

The effect of feeding substances which cause liver-injury :

Glucuronic acid is known to take part in the process of conjugation whereby those toxic materials, which cause injury to the liver, are eliminated. Thus the administration of substances like camphor and chloral hydrate stimulates the liver to produce glucuronic acid in order to eliminate them as conjugated glucuronides. As we have suggested before, ascorbic acid may have a similar function in the elimination of certain toxic metabolities in diphtheria (1), tetanus (2) and tuberculosis (5). Experiments have now been carried out to investigate the effect of feeding camphor and chloral hydrate on the urinary excretion of free as well as combined ascorbic acid.

(i) Camphor :

100 Mg. of camphor dissolved in cocoanut oil (1 c.c.) were fed daily to guinea-pigs (on normal diet). The results show that the excretion of free ascorbic acid in the urine remains practically the same while the combined ascorbic acid excretion is considerably increased (Table III).

TABLE III

Mg. of ascorbic acid excreted per animal per day before and after camphor administration.

No. of Expt.	No. of days	Free ascorbic acid		Combined ascorbic acid	
		Before	After	Before	After
I	1	0.169	0.221	0.008	0.028
	2	0.229	0.251	0.005	0.068
	3	0.348	0.235	0.000	0.172
	Mean	0.248	0.235	0.004	0.089
II	1	0.201	0.184	0.071	0.000
	2	0.201	0.221	0.000	0.027
	3	0.261	0.248	0.000	0.103
	Mean	0.220	0.217	0.023	0.043
III	1	0.210	0.204	0.042	0.207
	2	0.186	0.228	0.047	0.000
	3	0.234	0.235	0.000	0.217
	Mean	0.210	0.222	0.029	0.141

No. of Expt.	No. of days	Free ascorbic acid		Combined ascorbic acid	
		Before	After	Before	After
IV	1	0.216	0.200	0	0.106
	2	0.209	0.204	0	0.201
	3	0.215	0.197	0	0.300
	Mean	0.213	0.200	0	0.269

(ii) Chloral hydrate :

Chloral hydrate, when administered orally in effective doses, is known to cause an appreciably increased elimination of glucuronide in the urine. 100 Mg. of chloral hydrate dissolved in water (0.25 c.c.) were fed to normal guinea-pigs and the urinary excretion of ascrobic acid was determined. The results (Table IV) indicate that ascorbic acid does not take part in eliminating chloral hydrate as there was an appreciable decrease of combined ascorbic acid in the urine with simultaneous increase in free ascorbic acid.

TABLE IV

Mg. of ascorbic acid excreted per animal per day before and after chloral hydrate administration.

No. of Expt.	No. of days	Free ascorbic acid		Combined ascorbic acid	
		Before	After	Before	After
I	1	0.100	0.159	0.278	0.058
	2	0.128	0.109	0.247	0.172
	3	0.125	0.100	0.162	0.158
	Mean	0.117	0.122	0.229	0.129
II	1	0.079	0.245	0.358	0.018
	2	0.100	0.150	0.267	0.064
	3	0.123	0.182	0.342	0.068
	Mean	0.100	0.192	0.322	0.050
III	1	0.171	0.339	0.485	0.059
	2	0.159	0.225	0.417	0.056
	3	0.159	0.176	0.306	0.173
	Mean	0.163	0.246	0.402	0.096
IV	1	0.183	0.162	0.240	0.180
	2	0.143	0.208	0.191	0.050
	3	0.160	0.208	0.121	0.125
	Mean	0.162	0.190	0.214	0.119
V	1	0.155	0.111	0.245	0.208
	2	0.166	0.160	0.128	0.076
	3	0.144	0.136	0.176	0.089
	Mean	0.155	0.135	0.183	0.124

The effect of feeding inorganic toxic substances :

Inorganic substances like copper salts, fluorides and phosphorus are sufficiently toxic to kill the animal. The effect of administration of such substances in sublethal doses on the urinary excretion of free and combined ascorbic acid was expected to throw some light on the mechanism of detoxication effected by ascorbic acid.

(i) *Copper as cupric chloride :*

2.5 Mg. of copper as cupric chloride dissolved in water (0.5 c.c.) were administered orally to normal guinea-pigs and the effect on the urinary excretion of both free and combined ascorbic acid was investigated in the usual manner. Table V shows a decrease in free as well as combined ascorbic acid.

TABLE V

Mg. of ascorbic acid excreted per animal per day before and after $CuCl_2$ administration.

No. of Expt.	No. of days	Free ascorbic acid Before	Free ascorbic acid After	Combined ascorbic acid Before	Combined ascorbic acid After
	1	0.300	0.104	0.090	0.087
I	2	0.228	0.171	0.091	0.082
	3	0.272	0.306	0.009	0.000
	Mean	0.266	0.193	0.063	0.058
	1	0.294	0.107	0.110	0.164
II	2	0.186	0.103	0.134	0.000
	3	0.118	0.100	0.206	0.000
	Mean	0.199	0.103	0.150	0.054
	1	0.193	0.183	0.116	0.000
III	2	0.339	0.085	0.000	0.040
	Mean	0.263	0.134	0.058	0.020
	1	0.311	0.172	0.104	0.061
IV	2	0.267	0.200	0.018	0.000
	Mean	0.289	0.186	0.061	0.030
	1	0.252	0.280	0.034	0.011
V	2	0.233	0.219	0.132	0.063
	Mean	0.241	0.249	0.086	0.037

(iii) *Phosphorus :*

When phosphorus (about 2 mg.) in water suspension (0.2 c.c.) was fed to normal guinea-pigs they behaved in a similar manner to those suffer-

ing from copper intoxication. The results (Table VI) show that there is no increased elimination of combined ascorbic acid. .

TABLE VI

Mg. of ascorbic acid excreted per animal per day before and after phosphorus administration.

No. of Expt.	No. of days	Free ascorbic acid		Combined ascorbic acid	
		Before	After	Before	After
I	1	0.100	0.100	0.316	0.200
	2	0.121	0.127 .	0.295	0.073
	Mean	0.110	0.113	0.305	0.136
II	1	0.159	0.145	0.094	0.128
	2	0.164	0.121	0.288	0.098
	Mean	0.161	0.133	0.191	0.113
III	1	0.151	0.100	0.188	0.082
	2	0.100	0.128	0.234	0.138
	Mean	0.125	0.114	0.211	0.110

Fluoride as NaF :

The effect of fluoride poisoning in guinea-pigs on the urinary excretion of both free and combined ascorbic acid has been studied. 20 Mg. of sodium fluoride (0.25 c.c.) were fed to normal guinea-pigs and the urinary excretion of ascorbic acid was determined in the usual manner. The results (Table VII) are inconclusive as uniform results were not obtained, but it seems that there is no increase in the excretion of combined ascorbic acid.

TABLE VII

Mg. of ascorbic acid excreted per animal per day before and after NaF administration.

No. of Expt.	No. of days	Free ascorbic acid		Combined ascorbic acid	
		Before	After	Before	After
I	1	0.205	0.209	0.071	0.067
	2	0.200	0.245	0.112	0.137
	3	0.288	0.200	0.000	0.000
	Mean	0.231	0.217	0.061	0.068
II	1	0.171	0.325	0.117	0.108
	2	0.288	0.200	0.000	0.000
	3	0.216	0.226	0.044	0.000
	Mean	0.225	0.250	0.050	0.036

No. of Expt.	No. of days	Free ascorbic acid Before	After	Combined ascorbic acid Before	After
III	1	0.288	0.236	0.118	0.110
	2	0.312	0.206	0.038	0.010
	3	0.248	0.206	0.111	0.020
	Mean	0.281	0.219	0.089	0.047
IV	1	0.291	0.146	0.159	0.118
	2	0.325	0.107	0.175	0.091
	3	0.354	0.155	0.129	0.036
	Mean	0.320	0.136	0.154	0.081
V	1	0.183	0.115	0.146	0.162
	2	0.122	0.200	0.254	0.152
	3	0.118	0.200	0.236	0.064
	Mean	0.141	0.171	0.212	0.117
VI	1	0.218	0.181	0.315	0.156
	2	0.171	0.200	0.353	0.142
	3	0.195	0 100	0.205	0.157
	Mean	0.194	0.160	0.291	0.151

The effect of injecting potassium cyanide :

Cyanide is known to undergo detoxication by being changed into thiocyanate—the thio-constituents of the body supplying the necessary sulphur. Experiments by Leibowitz and Guggenheim (10-11) have shown that within certain limits the effect of potassium cyanide can be neutralised by previous contact with ascorbic acid in experiments with mammals and bacteria. The detoxicating effect was greater the longer the substance had previously been in contact. This was further supported by the observation that ascorbic acid increases the minimum lethal dose of cyanide for rats and mice. Moreover potassium cyanide in low concentrations has some protective action against oxidation of ascorbic acid ; at high concentrations destruction was actually accelerated (12). This protective action of the cyanide along with the cryoscopical determination of ascorbic acid-potassium cyanide mixture led Leibowtiz and Guggenheim to suppose the formation of a chemical compound of ascorbic acid with hydrocyanic acid. These observations led us to investigate the effect of potassium cyanide injection on the urinary output of both free and combined ascorbic acid. 0.5 Mg. of a freshly prepared solution (0.5 c.c. water) of potassium cyanide (which by trial was found to be sublethal) was injected subcutaneously into normally-fed guinea-pigs and the urinary excretion was examined before and after the cyanide administration (Table VIII). The excretion of free ascorbic acid was increased, but uncertain values were obtained for combined ascorbic acid.

TABLE VIII

Mg. of ascorbic acid excreted per animal per day before and after cyanide injection.

No. of Expt.	No. of days	Free ascorbic acid		Combined ascorbic acid	
		Before	After	Before	After
I	1	0.126	0.162	0.150	0.160
	2	0.121	0.185	0.130	0.130
	3	0.111	0.183	0.090	0.173
	Mean	0.119	0.176	0.126	0.152
II	1	0.268	0.169	0.192	0.150
	2	0.132	0.291	0.248	0.139
	3	0.125	0.219	0.190	0.171
	Mean	0.155	0.226	0.210	0.153
III	1	0.185	0.205	0.143	0.210
	2	0.176	0.238	0.119	0.070
	3	0.171	0.229	0.170	0.170
	Mean	0.177	0.224	0.144	0.153

The effect of toxic substances on the ascorbic acid content of the tissues of guinea-pigs :

It has been observed that during intoxication, ascorbic acid metabolism is disturbed and a marked depletion of the ascorbic acid content of tissues of normally fed guinea-pigs occurs when the animals are injected with sub-lethal doses of diphtheria and tetanus toxins (1-2). It was also observed that the adrenal gland suffered a maximum depletion of about 50 per cent in ascorbic acid. Investigations have now been carried out on the effect of the administration of toxic substances like histamine, phenol, camphor, chloral hydrate, copper, phosphorus, fluoride and cyanide on the ascorbic acid content of the tissues of guinea-pigs. The animals under investigation were kept on green grass and germinated gram. The different kinds of toxic materials were administered daily either orally or by injection as indicated in Table IX below, for 3 consecutive days. On the fourth day each animal was killed by a blow on the head and the ascorbic acid values of the organs, like adrenal, liver and kidney were determined by the method described by Ghosh and Guha (13) and Sen-Gupta and Guha (14—15). The ascorbic acid contents of these organs of guinea-pigs which were treated with different toxic materials showed a depletion of free ascorbic acid content in these organs when compared with that of controls.

TABLE IX

No. of Expt.	No. of animals employed.	Nature and quantity of toxic material.	Mode of administration: daily for 3 consecutive days.	Mean values of ascorbic acid (in mg.) per g. of the tissue.		
				Adrenal	Liver	Kidney
1	4	Camphor (100 mg.) dissolved in cocoa-nut oil (0.5 c.c.)	Orally	0.333	0.156	0.080
2	6	Chloral hydrate (100 mg.) in water	,,	0.146	0.074	0.056
3	6	Phenol (5 mg.) in water	,,	0.229	0.093	0.068
4	5	Copper chloride (CuCl₂) (5 mg.) in water	,,	0.199	0.096	0.062
5	3	Red phosphorus (2 mg.) in water sus-pension	,,	0.171	0.080	0.056
6	6	Sodium fluoride (20 mg.) in water	,,	0.119	0.116	0.061
7	3	Histamine hydro-chloride (0.8 mg.) in water	Injection	0.269	0.171	0.079
8	3	Potassium cyanide (0.5 mg.) in water	,,	0.315	0.169	0.078
9	3	Controls		0.346	0.177	0.088

DISCUSSION AND SUMMARY

An investigation on the effect of various toxic substances on the urinary excretion of both free and combined ascorbic acid has been carried out in order to throw light on the function of ascorbic acid in the process of detoxication.

1. Injection of histamine dihydrochloride caused similar increased elimination of combined ascorbic acid as in cases with the injection of sublethal doses of diphtheria and tetanus toxins with a slight decrease in free ascorbic acid excretion.

2. Ingestion of phenol seemed to cause increased elimination of combined ascorbic acid in the urine with considerable decrease in free ascorbic acid.

3. Ingestion of camphor caused an increased urinary excretion of ascorbic acid without any appreciable change in the free ascorbic acid excretion.

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4. Other substances like chloral hydrate, copper chloride, red phosphorus, sodium flouride and potassium cyanide caused decreased elimination of combined ascorbic acid in the urine.

It thus seems that in the case of poisoning by histamine, phenol and camphor as by certain bacterial toxins, the mechanism of detoxication involves excretion of some ascorbic acid in a combined state. With chloral hydrate, copper, phosphorus, fluoride and cyanide similar elimination of combined ascorbic acid does not occur. Thus the detoxicating capacity of ascorbic acid would seem to be selective, inorganic poisons being in general unaffected by it. Further work is in progress.

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NICOTINIC ACID CONTENT OF FISH

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Since the effect of nicotinic acid in the treatment of the disease of black tongue in dogs and pellagra in man had been established, the problem of quantitative estimation of the substance in question in various biological materials has attracted considerable attention. The different methods adopted by different workers are briefly discussed below.

Vilter *et el* (1) have devised a colorimetric reaction for the determination of nicotinic acid and nicotinamide and other pyridine derivatives. This method is based on the colour reaction of pyridine with 2:4-dinitrochlorobenzene and alkali hydroxide (2). This method is much less sensitive and more tedious than the other methods. Moreover, the decolorisation of the concentrated filtrate before colorimetric estimation which is involved in this method might lead to some loss due to the adsorption of nicotinic acid.

Covello (3) has modified Vongerichten's method by using 3:4-dinitrochlorobenzene instead of 2:4-dinitrochlorobenzene but the difficulties encountered by the former method remained practically unsolved.

The colorimetric method used by Porje (4) is based on König's (5) observation that a yellow colour develops when pyridine reacts with cyanogen bromide and a primary or secondary aromatic amine.

Aniline has been used by Swaminathan (6) which produces a colour reaction of the type described by König. Bandier and Hald (7) used metol instead of aniline.

The growth promoting action on various micro-organisms has been adopted for the quantitative estimation of nicotinic acid by Querido *et el* (8).

The present communication deals with the results concerning the mean values of nicotinic acid content of the muscle tissue of 20 varieties of Bengal fish obtained from analyses of 4 to 5 samples of each kind. The method adopted is that of Swaminathan (6) slightly modified.

EXPERIMENTAL

50 G. of finely minced fish tissue were extracted with 100 c.c. of boiling water for 30 minutes. After cooling and centrifuging, the residue was extracted twice in a similar way with 50 c.c. portions of water but the heating was carried out for 10 minutes. The washings were added to the original filtrate and the whole was evaporated down to 100 c.c. on water bath. The extract was cooled to room temperature, 5 c.c. of 20% trichloroacetic acid were added and the precipitated protein was removed by centrifuging. To the clear filtate, concentrated hydrochloric acid was added to make about 5% acid. The solution was evaporated on water bath to a volume of 40 c.c. and thereby any nicotinamide present was converted into the corresponding

acid. This was cooled and adjusted to pH 7.0 with concentrated sodium hydroxide solution. The protein precipitated at this pH was removed by centrifuging. The volume of the extract was then made up to 50 c.c. ; aliquots (10 c.c.) were taken in a 25 c.c. graduated flask ; two drops of a freshly prepared saturated aqueous aniline solution and 4 c.c. of a freshly prepared aqueous cyanogen bromide (1%) solution were then added. The volume was made up to 25 c.c. and the mixture was shaken and allowed to stand for 2—3 minutes. The bright yellow colour which developed was compared in a colorimeter with a standard similarly prepared with a known amount of nicotinic acid. The estimation should be finished within 30 minutes.

A blank experiment was always made with the original extract to eliminate the effect of the slight yellow colour which develops during hydrolysis. The results obtained are given in Table I.

·It was noted that an intense yellow colour develops on concentrating the extract at pH 7.0 due to interfering substances other than nicotinic acid and its derivatives, which hinders the colorimetric estimation and 'the colour once produced cannot be removed completely even by charcoal treatment. Hence the acid solution should be concentrated to a small bulk before adjustment of the pH to 7.0.

In order to compare the results obtained by our modification with those obtained by Swaminathan's method, estimations have been carried out on the same sample of tissue by both the methods and the results are given in Table II.

TABLE I

Values for nicotinic acid are given in mg. per 100 g. of fresh fish muscle tissue.

Bengali name.	Zoological name.		Nicotinic acid.
Magur	Clarius batrachus	...	... 1.02
Lata	Ophicephalus punctatus	...	... 1.00
Boal	Wallago attu	...	... 0.98
Dhain	Silonia silundia	...	... 0.91
Katla	Catla catla	...	... -0.82
Shinghi	Saccobranchus fossilis	...	... 0.81
Koi	Anabas testudineus	...	... 0.79
Parsey	Mugil parsia	...	... 0.77
Hilsa	Clupea ilisa	...	... 0.76
Mrigal	Cirrhina mrigala	...	... 0.73
Rohu	Labeo rohita	...	... 0.68
Royna	———	...	... 0.62
Bacha	Chpisoma garua	...	... 0.61
Bhole	Sciacna coitor	...	... 0.53
Shole	Ophcephalus striatus	...	... 0.52
Air	Arius arius	...	... 0.50
Mourala	Amblypharyn godonmola	...	... 0.48
Chingri	———	...	... 0.43
Pakal	———	...	... 0.43
Bele	Glassgobius giuris	...	... 0.32

TABLE II

*Values for nicotinic acid are given in mg. per 100 g. of
fresh fish muscle tissue.*

Bengali name.	Swaminathan's method. (nicotinic acid)	Modified method. (nicotinic acid)
Rohu	0.52	0.58
Katla	0.76	0.81
Air	0.46	0.48
Parsey	0.66	0.72
Magur	1.10	1.12

From the above results it is clear that the method used by Swaminathan always gives slightly lower results than those obtained by the modified method. It is probable that in the process of decolorisation of the concentrated filtrate with charcoal there is a slight loss due to adsorption. Furthermore, repeated centrifugations may be one of the causes of these low results.

Recovery of added nicotinic acid :—

In testing whether our method would permit added nicotinic acid to be recovered quantitatively, the following results were obtained, which are quite satisfactory (Table III).

TABLE III

*Values for nicotinic acid are given in mg. per 100 g. of
fresh fish muscle tissue.*

Sample no.	Nicotinic acid added.	Nicotinic acid in the original tissue.	Total acid obtained.	Acid recovered.
1.	0.10	0.23	0.32	0.09
2.	0.15	0.20	0.35	0.15
3.	0.20	0.34	0.52	0.18
4.	0.25	0.29	0.53	0.24

Comparing this modified method with Swaminathan's it seems that:—

(1) the latter method is somewhat more laborious and takes a longer time,

(2) decolorising the concentrated filtrate with charcoal and repeated centrifugation may lead to slightly low results, and

(3) it is rather difficult to adjust the quantity of solid lead acetate used for the precipitation of protein. Sometimes, it becomes difficult to get the clear filtrate. During hydrolysis, the colour produced is so intense that it cannot be completely removed even by treatment with charcoal.

Preparation of cyanogen bromide :—

A solution of 65 g. of potassium cyanide in 120 c.c. of water is cooled to 0° and slowly added to 150 g. of bromine, also cooled to 0° ; and the liquid is agitated while mixing. The product is then distilled at 60°-70° and the evolved cyanogen bromide condensed. The yield is 90%.

SUMMARY

From the above results it is found that Magur (*Clarius batrachus*) has the highest nicotinic acid content, viz., 1.02 mg. per 100 g. of fresh muscle tissues ; then comes Lata (*Ophicephalus punctatus*) having 1.00 mg., and the poorest source of nicotinic acid is Bele (*Glasgobius giuris*) which gives a figure of 0.32 mg.

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COPPER CONTENT OF FISH

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The investigation of Hart, Steenbock, Waddel and Elvehjem (1) first demonstrated the importance of copper as a supplement to iron for hæmoglobin formation in anemic rats. Their work showed definitely that in the presence of copper, soluble inorganic salts of iron can be used directly for hæmoglobin formation. Since that time a number of workers have studied the factors affecting hæmoglobin production in rats rendered anemic by a whole milk diet. Most of this work has verified the original conclusions concerning the importance of copper and to-day, although there are some who still feel that copper is not the only element in addition to iron which possesses hematopoietic properties, practically all workers agree that copper is an active agent in hæmoglobin synthesis.

While the action of copper is catalytic in the sense that it does not appear as a constituent of the hæmoglobin formed, yet the action of copper in hæmoglobin formation and regeneration is now regarded not as a drug effect but as a part of the normal nutritional process. But the exact mechanism involved in the function of copper in hæmoglobin formation has not yet been ascertained.

The present communication gives the results concerning the mean values of copper of the different varieties of Bengal fish, obtained usually from analyses of 4 to 7 samples of each kind. The zoological names of a few of these have not been obtained.

EXPERIMENTAL

The method used was that of Callan and Henderson (2) as modified by McFarlane (3). In this method sodium diethyl-dithiocarbamate is added to the solution under test ; a yellow complex with copper is formed and this is extracted by shaking with amyl alcohol. The extract is compared in a colorimeter. The reaction, although independent of pH, is carried out in alkaline solution in the presence of pyrophosphate in order to prevent interference by iron.

In this method the copper present in the fish tissue was directly extracted with trichloroacetic acid according to Tompsett (4) which gave accurate and rapid separation of copper. The results obtained were compared with those obtained after digestion with sulphuric and perchloric acids and the results obtained by both the methods were found to be in good agreement. The added iron was quantitatively recovered by direct extraction with trichloroacetic acid.

Direct extraction with trichloroacetic acid :—

Ten g. of the finely minced fish were taken in a glass mortar, ground up with washed sea-sand, 20 c.c. of 20% trichloro-acetic acid were added and the grinding continued till an uniform mixture was obtained. The mass was then centrifuged and the clear liquid decanted off into a 250 c.c. beaker. The residue was washed three times with 10% trichloro-acetic acid and centrifuged each time. The filtrate and washings were mixed together and filtered through acid-washed filter paper. The clear filtrate thus obtained was made up to 50 c.c. ; 5 c.c. of 4% sodium pyrophosphate were added followed by concentrated ammonia until faintly alkaline to litmus. Ten c.c. of amyl alcohol were added and then 2 c.c. of 2% sodium diethyl-dithiocarbamate solution and the mixture well shaken. The golden yellow colour developed was compared with a standard similarly prepared. Table I gives the results obtained by the above method.

TABLE I

Values of copper are given in mg. per 100 gms. of raw fish muscle.

Bengali name.	Zoological name.		Copper content.
Koi	Anabas testudineus	...	... 0.162
Air	Arius arius	...	... 0.064
Shinghi	Saccobranchus fossilis	...	... 0.153
Bele	Glassgobius giuris	...	... 0.031
Shole	Ophcephalus striatus	...	... 0.112
Hilsa	Clupea ilisa	...	... 0.140
Parsey	Mugil parsia	...	... 0.102
Mowrala	Amblypharyn godonmola		... 0.073
Bhetki	Lates calcifer	...	... 0.106
Kharsala	————	...	... 0.134
Mrigal	Cirrhina mrigala	...	... 0.121
Pangas	Pangasius pangasius	...	... 0.080
Rohu	Labeo-rohita	...	... 0.125
Bam	————	...	... 0.060
Sarputi	Barbus sarana	...	... 0.077
Pabda	————	...	... 0.093
Royna	————	...	... 0.053
Dhain	Silonia silundia	...	... 0.078
Bacha	Chpisoma garua	...	... 0.112
Pakal	————	...	... 0.061
Bhole	Sciacna coitor	...	... 0.053
Boal	Wallago attu	...	... 0.082
Katla	Catla catla	...	... 0.122
Magur	Clarius batrachus	...	... 0.148

Acid digestion method :

The method adopted was that of McFarlane (3) as slightly modified by Tompsett (5).

Five g. of the minced fish were taken in a Kjeldahl flask with 5 c.c. of concentrated nitric acid. The mixture was digested on a boiling waterbath until the contents of the flask were almost dry. Then the remaining organic matter was decomposed, by heating over a flame with 2 c.c. of concentrated sulphuric acid and 3 c.c. of perchloric acid. The contents of the flask were diluted to 10 c.c. with distilled water and 5 c.c. of 4% sodium pyrophosphate solution-added. The solution was treated with ammonia until slightly alkaline to litmus and then placed on a waterbath at 80° for 15 minutes. After cooling 10 c.c. of amyl alcohol were added followed by 2 c.c. of 2% sodium diethyl-dithiocarbamate solution and the whole was well shaken. The final estimation was done as before. The comparative results of both the methods when applied to the same sample of fish tissue are given in Table II.

TABLE II

Values of copper are given in mg. per 100 gms. of raw fish muscle.

Sample No.	Direct extraction with tri-chloro-acetic acid.	Digestion by sulphuric and perchloric acids.
1.	0.137	0.132
2.	0.082	0.081
3.	0.112	0.108
4.	0.121	0.126
5.	0.093	0.100
6.	0.140	0.143

The amounts of recovery of the added iron by trichloro-acetic acid extraction are indicated in Table III.

TABLE III

Values of copper are given in mg. per 100 gms. of raw fish muscle.

Sample No.	Copper added.	Total copper as estimated by tri-chloro-acetic acid.	Copper in the original sample by difference.	Copper in the original sample by estimation.
1.	0.02	0.121	0.101	0.100
2.	0.05	0.172	0.122	0.122
3.	0.05	0.160	0.110	0.106
4.	0.05	0.133	0.083	0.081
5.	0.05	0.120	0.070	0.071

SUMMARY

Table I gives the mean values of copper in 24 different varieties of fish, obtained usually from analyses of four to seven samples of each kind. Among the fish studied Koi (*Anabas testudineus*) has the highest copper content, namely 0.162 mg., then come Shinghi (*Saccobranchus fossilis*) with 0.153 mg. and Magur (*Clarius batrachus*) with 0.148 mg. per 100 g. These fishes have also been found to be good sources of available iron (6).

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· MINERAL CONSTITUENTS OF HUMAN HAIR*

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In connection with an investigation on the lead content of human tissues it was discovered that human hair is very rich in lead—as much as 508 gm. of lead (per kilo) was found in the black hair of a Hindu woman. To determine if hair possesses a special affinity for poisonous metals like lead or if it behaves exactly in the same way towards other metals, it was subjected to careful analysis and was found to contain, in much excess, all the metals which are likely to be present in human tissues.

Boyd and De (1) showed by spectrographic method that minor elements, such as rubidium and vanadium, as well as the common metals, are always present in our system. Attention was therefore directed to these metals and also to other metallic elements which are known to be normal constituents of human tissues.

METHODS OF ANALYSIS

For determination of organic matter the usual method of oxidation in a combustion furnace was adopted, and for destruction of organic matter for liberating the metals, three different methods were employed—(1) wet oxidation (Ramberg's method, 12) by HNO_3 and H_2SO_4 for Pb, As, Cu, Zn, etc., (2) ordinary ashing in a muffle furnace for Mn, Ni, Co, Fe, Al, Si, Ca etc., and (3) fusion with KNO_3 and Na_2CO_3 for phosphorus, sulphur etc.

Lead was determined by the dithizone method (colorimetric) of Roche Lynch, Slater and Osler (2) slightly modified by Bagchi and Ganguly (3) ; arsenic by Gutzeit method, modified by Davis and Maltby (4) ; copper by sodium diethyl-dithiocarbamate method (colorimetric) of Tompsett (5) with slight modification, and zinc by the method of Sylvester and Hughes (6) in which the final titration was made with standard potassium ferrocyanide solution in presence of diphenyl benzidine.

For determination of manganese the periodate method of Willard and Greathouse modified by Richards (7) and also the method of Skinner and Peterson (8) were adopted according to circumstances. Cobalt was determined by a colorimetric method of Mayr and Feigl (9) using alpha-nitroso-beta-naphthol reagent and nickel by the usual gravimetric method in which dimethyl-glyoxime is used (tartrate solution being added to prevent precipitation of iron by ammonia).

The other elements were determined by the usual quantitative methods of analysis.

The following table gives the analytical figures of hair obtained from four different sources—one of them being a hair-cutting saloon frequented by Indian gentlemen and this sample was a mixture of hair of about thirty male adults. Every sample was thoroughly cleaned by treatment, successively, with running water, hot soap solution, dilute caustic alkali, hot dilute hydrochloric acid, distilled water, alcohol and ether. Further treatment with ammonium acetate followed by repeating the above method did not effect further improvement and attempts, on the other hand, to reduce the number of steps for simplifying the method did not prove successful.

TABLE I.

	European girl aet. 14. Brown hair.	Hindu woman aet. 25. Black hair.	Mixed hair of 30 male adults —from a hair cutting saloon.	Hindu male aet. 28. A case of lead poisoning.
C	44.03%	44.20%	44.60%	43.80%
N	13.70%	13.68%	14.60%	14.20%
H	5.58%	5.60%	5.40%	6.10%
S	3.80%	1.50% (?)	3.80%	4.20%
P	0.065%	0.096%	0.08%	0.098%
Cl	1.98%	2.00%	2.00%	2.40%
Water	3.96%	4.20%	4.10%	4.20%

Milligrammes per kilo.

Pb.	21.0	284.0	47.7	241.0
Cu.	64.0	62.8	108.0	28.8
As.	2.4	2.2	2.2	1.8
Zn.	116.0	182.0	212.0	420.0
Fe.	133.0	126.0	141.0	170 0
Mn.	28.4	25.0	38.0	46.0
Co.	14.2	16.0	18.1	16.4
Ni.	5.4	5.5	8.2	6.7
Ca.	212.0	188.0	208.4	267.2
Al.	26.0	26.0	32.0	36.0
Si.	188.0	178.6	150.4	164.5
Bi.	Nil	Nil	Nil	Nil
Ag.	Nil	Nil	Nil	Nil
Sb.	Nil	Nil	Nil	Nil
Hg.	Nil	+	Nil	Nil

N.B. Mg, I and Sn are also present but their quantities could not be determined for want of sufficient materials. This portion of the investigation is still in progress.

It is evident from Table I that the amounts of C, H, N, S and P present in hair are practically of the same magnitude in every sample irrespective of age, sex and nationality. As hair is related histologically to the skin and nail, the proportions of these elements in hair indicate the possibility of presence of same types of proteins as in the latter tissues. On the other hand, there is enough scope for speculation about the metallic portion of its composition. It is not known in which

form they are present in hair, or for the matter of that, in other tissues of the body. Possibly it is present in combination with proteins or in both organic and inorganic forms.

The variability in the amounts of the metallic elements in hair appears to be a remarkable feature. At first we thought it was due to experimental errors but duplicate experiments and recovery of added amounts from the same sample convinced us of the possibility of such wide differences. A similar variation in the case of poisonous metals such as arsenic and lead has also been observed in different tissues of the same individual and in the same tissue of different individuals (Bagchi and Ganguly, 3, 10). It may therefore be presumed that healthy tissues behave exactly in the same way towards other metals as they do towards arsenic and lead. The following pertinent questions may therefore be asked in this connection— (1) Is this variation an accident? (2) Are the metals, after their introduction into the system possibly with food, retained temporarily in the tissues for subsequent elimination? (3) If not, are they required by the system to carry on the various physiological functions of the body? (4) Do they mainly act as catalysts? (5) Are the poisonous metals, such as arsenic, capable of acting as catalytic poisons on the numerous organic catalysts or enzymes present in various tissues of the animal body and establishing an equilibrium between the antagonistic systems of enzymes? These are the problems that require careful investigation by the biochemists.

Whatever be the case, such variations do occur in the tissues and consequently the hair in which the metals are dumped for elimination as suggested elsewhere (Bagchi ct al, 11, 12), must necessarily show individual variation with regard to the metals it contains.

Of all the metals detected in hair, a careful investigation was made about lead only. Nearly 200 samples of hair from persons of all ages, sex and nationality were analysed and the following observations have been recorded (Bagchi et al, 10).

TABLE II.

	Minimum.	Maximum.
Deep black hair (Bengali women)	170.0	508.0
Brown, auburn and other shades (Europeans) ...	9.0	21.0
Grey (Europeans and Indians)	3.0	21.0

The table shows that the black hair is rich in lead while hair of other shades of colour and also the grey hair, contain the minimum amounts of lead. It is, therefore, a matter for careful investigation if lead is responsible for the characteristic black pigment which imparts fine black colour to hair of Bengali women and also of people living in the tropical countries. Apart from the fact that it is eliminated through hair as we suggested in a previous communication (loc. cit), lead appears to play some other rôles while present in hair. It is known that blonde women with

auburn hair cannot stand exposure, especially of their head, to the sun while people with black hair, as in our country, can stand such exposures without any difficulty while working in the sun for hours together. It may be enquired if hair, rich in lead, act as a protective cap against certain rays of the sun just in the same way as gloves and aprons impregnated with lead protect the radiologists when they expose themselves to X-rays. It is likely that more we are exposed to the sun, more lead is excreted into the hair, and it may, therefore, be suggested further that people wearing no head dress and exposed to the tropical sun, as in the case of the Bengalis, may possibly retain more lead in their hair than those who put on head dresses. The following table is likely to lend support to this possibility.

TABLE III.

	Men	Women
Europeans (resident in Calcutta) including Jews and Anglo-Indians	20.8	18.4
Bengalis (Hindus and Muslims)	34.5	115.6
Punjabis	20.2	45.5
Madrassis	22.7	—
U. P. and Beharis	21.6	—

In conclusion, it may be stated that as all metallic elements are found in hair in large excess, much larger than in the tissues, hair may be taken as an index of the human tissues with regard to the metals which are introduced into the system and retained by the various organs. The liver and the kidney are the organs usually selected for detection as well as for determination of the mineral constituents of the body but they pale into insignificance before hair. It may be stated, without much fear of contradiction, that hair is the most suitable tissue for such investigations and it is sure to give, in the hands of competent biochemists and expert spectroscopists, all sorts of information connected with the proportions of metallic constituents of our body and possibly of their significance.

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BOWEL DISORDERS IN BENGAL FROM THE LABORATORY POINT OF VIEW

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(Received for publication, November 11, 1940)

Bowel disorders include a long list of ailments, of which only those of infective origin will come under the scope of this review. These disorders of the intestinal tract including acute, sub-acute and chronic diarrhœa, dysentery and what is popularly known as dyspepsia with symptoms of indigestion, vague abdominal pain or even colic, belong to a group of disorders for which practitioners are very frequently consulted. These bowel diseases cause a great deal of drainage of health and vitality of the people of Bengal, particularly in the rural areas. The morbidity and mortality due to these ailments are very high. Their ill effects would appear to be higher still if one takes into consideration their debilitating effect, which renders the patient easily susceptible to the secondary infections.

It would, therefore, be interesting to survey these cases from the etiological point of view, which can be best studied by examining the samples of stool from the patients, whose history of illness and signs and symptoms suggest existence of some pathological condition in the gastro-intestinal tract. And as such examinations are best likely to demonstrate the causative agents and also their relative incidence, this review is devoted to the analysis of the result of examination of samples of stools from cases of bowel disorders, where their physicians thought that such examinations were essential for proper diagnosis of the causative factor. Here is the analysis of the findings of our investigation carried out at the diagnostic section.

TABLE I

Analysis of microscopical examination of stool.

Number of cases examined.	Number of cases where causative agents were detected on microscopical examinations.	Percentage of positive causes.
1,376	665	48.3

It will be seen from Table I that in 48.3% of cases, whose stools were sent for examination, pathogenic organisms were detected by microscopical examination. Of the negative cases, a fair number showed the presence of Charcot-Leyden crystals, pus cells, R.B.C., etc. giving indication of the existence of pathological conditions. Thus of the 711 negative cases 36 showed the presence of Charcot-Leyden crystals in the stool and in many of the samples, pus cells, R.B.C., etc., were found, which indicated the necessity of cultural examination and re-examination of a second sample of stool from the same patient, preferably following a dose of purgative. Apart

from the detection of the causative organisms these examinations are likely to reveal the condition of the digestive functions of the patients regarding carbohydrate, fat and protein.

TABLE II

Relative proportion of offending organisms in positive cases as found in microscopical examination only.

Total number of positive cases.	Vegetative form or cyst of Entamoeba histolytica.	Vegetative form or cyst of Giardia Lamblia.	Vegetative form or cyst of Trichomonas Intestinalis.	Ova of Trichuris Trichiura.	Ova of Ankylostoma Duodonalis.	Larva and ova of Strongyloides Stercoralis.	Ova of Ascaris Lumbricoides.	Ova of Hymenolepis Nana.	Ova of Oxyuris Vermicularis.	Balantidium Coli.	Ova of Tania Saginata.
Number 665	327	150	119	21	15	15	10	4	2	1	1
Percentage	49.1	22.5	17.8	3.1	2.2	2.2	1.5	0.6	0.3		

The analysis of Table II will show that of the pathogenic organisms of non-bacterial group, infection with *Entamoeba histolytica* is the commonest, being about 49.1% of the positive cases. Next in order come intestinal flagellates, which were isolated from 40.3% of positive cases. It would be remembered that cases with multiple infection are quite common. Thus in a single sample of stool one frequently finds, multiple infections of *Entamoeba histolytica*, flagellate and helminthic ova. The same stool on culture may show the growth of non-lactose fermenters. These facts are not properly taken notice of. Thus when a case comes for dysentery or diarrhoea one thinks it either of amœbic or bacillary origin, but it would be seen from the above table that the incidence of flagellate infection with *Lamblia Intestinalis* or *Trichomonas* is very common. Again in a case of amœbic dysentery, often it will not suffice to treat the amœbic infection alone. Probably this fact contributes to one of the many causes of failure to cure completely a dysenteric infection, where amœba plays the most predominant rôle.

TABLE III

Result of cultural examination of stool.

Number of stool cultured.	Number of cases where offending bacteria were isolated.	Percentage of cases pathogenic bacteria were isolated.
571	288	50.5

It will be seen from the above table that pathogenic bacteria were isolated on culture in about 50% of the cases of stool, where no offending organisms of non-bacterial group could be detected. In the latter group amounting to 51.7%, it would appear that on an average 25.8% of the samples of stool sent for investigation showed the presence of offending bacteria alone. If added together it would appear that 74.1% of the samples of stool submitted for laboratory examination showed the presence of offending organisms either of bacterial or of non-bacterial origin. Although cultural examinations were not done in all the samples of stool, in which non-bacterial offending organisms were not detected and although the presence of pathogenic organisms in the stool of the patient alone is not sufficient to definitely establish the causal relationship between the organisms and the disease, the above analysis may be accepted as probably indication of the state of affairs in the bowel disorders of infective origin, as reviewed in our laboratory.

TABLE IV

Showing the proportional incidence of different types of bacteria in the stool of persons suffering from bowel disorders.

	Positive cases.	B. Salmonella.	B. Asiaticus.	B. Morgan.	Streptococcus.	B. Flexner.	B. Pyocianeus.	B. Shiga.	B. Proteus.	B. Dysentery strong.
Number of cases.	288	108	77	27	22	20	14	12	7	1
Percentage.		37.5	26.7	9.3	7.6	6.9	4.8	4.1	2.4	0.3

On culture of the stool occasionally more than one pathogenic micro-organisms were isolated from a single stool.

TABLE V

Stool culture of cases where non-bacterial offering organisms were detected.

Number of cases examined.	Number of positive cases.	Percentage of positive cases.
14	8	57

Although the number of cases examined in this table is very small, it gives some indications of a combined infection of bacterial and non-bacterial origin. It also suggests that in such cases, if treatment for the non-bacterial organisms does not produce the desired curative effect, the bacterial infection should also be treated.

It is the common experience of many that cases with dyspeptic symptoms have been treated even for years together with acid, enzymes and this, or that remedy, but when their stools were examined subsequently offending organisms were detected and were cured with a course of specific remedies in a short time. In some of these cases, before stool examinations, specific remedies were tried. And as one was not sure of the causative factors, the treatment was abandoned when the desirable effects of the therapeutic tests were not noticeable early.

It would be found from the analysis of stool examination that in roughly about 75% of samples of stool examined from cases of bowel disorders causative organisms could be found in the stool. In 48.3% of cases microscopical examination alone was sufficient to detect the cause and in 25.8% of cases cultural examination showed the pathogenic bacteria. Cases of multiple infection were not uncommon. All these suggest the necessity of adopting examination of stool both microscopically and culturally in order to arrive at a proper diagnosis of bowel disorders in this country.

SUMMARY

1. A very high percentage of cases of bowel disorders belonging to the group of diarrhœa, dysentery and dyspepsia in Bengal is of infective origin, which can be easily diagnosed by laboratory examination of the samples of stool from the patients.

2. Infection with intestinal flagellates is very frequent in this country.

3. Cases of infection with more than one organism are of common occurrence. This may be one of the reasons of frequent failure to cure bowel disorders completely.

4. The usefulness of routine laboratory examination of stool in bowel disorders has been suggested.

A COMPARATIVE STUDY OF THE BLOOD CONCENTRATIONS OF SULPHANILAMIDE AND SULPHAPYRIDINE

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While studying the effects of the sulphonamide group of drugs on the hæmopoietic system in the monkey, a study was made of the blood concentrations of sulphanilamide or *p*-aminobenzene-sulphonamide and the more recently discovered sulphapyridine, commonly known as M & B 693, whose real chemical name is 2-sulphanilyl-amino-pyridine. Although a number of workers, *e.g.*, Long and Fenistone (1), Stokinger (2), Schmidt and Hughes (3), Baines and Wien (4), Halpern *et al* (5) and more recently Marshall *et al* (6, 7) and Powell and Chen (8), have carried on investigations on the blood concentrations of sulphapyridine after giving the drug per os to animals, there still remains much to be done in this particular line of research. The discrepancies recorded in the literature for a long time, regarding the relative toxicity of sulphanilamide and sulphapyridine, have been primarily due to the absence of sufficient and reliable data regarding the blood concentrations of the drugs. The recent work of Marshall and his associates (9, 10, 11, 6, 7) has, however, brought to light the true nature of the absorption, distribution and elimination of the drugs following oral administration. In spite of the fact that sulphapyridine has now become a popular and very useful chemotherapeutic remedy, the phamacological data are still scanty. It is for this reason that the following results are communicated.

In the present work parallel comparisons have been made between sulphanilamide and sulphapyridine as regards their respective blood concentrations followinng oral administration and intramuscular injection in therapeutic doses.

METHODS

Animals and their treatment: —

Young healthy monkeys, weighing between 3.2 and 5.2 kg. were selected for our experiments. They were kept on a standard diet, consisting of gram 4 oz., green vegetables 4 oz., bananas 2, and placed under observation for a week, during which time their weights were periodically taken. Eight animals were finally chosen for our experiments. These were then divided into four groups of two animals each. The first group consisted of animals Nos. 7 and 8, the second group of Nos. 10 and 12. the third group of Nos. 11 and 13 and the fourth or last group of Nos. 4 and 14.

Administration of the drugs:—

Except in the case of group four, the drugs were given per os by stomach tube. All animals were fasted overnight and the drugs were administered in the morning in an empty stomach to ensure maximum absorption. The dose in each animal was repeated daily for a certain length of time in order to study the effects on the blood concentration of repeated administration of the drug. In the case of each animal the blood analyses were carried out on the first day the drug was given and then again after about a week. Following the administration of the drug, which was always done at the same hour each day, two c.c. samples of the blood were drawn out at fixed intervals from one of the leg veins by means of an accurately calibrated syringe.

The first group of monkeys received per os a daily dose of 0.06 gm. (No. 7) and 0.09 gm. (No. 8) per kg. of body weight of sulphanilamide (Prontosil Album, manufactured by Bayer, being used). The required quantities of the tablets were powdered and dissolved in water.

The second group were given orally a daily dose of 0.06 gm. per kg. of sulphapyridine (M & B 693), manufactured by May and Baker Co.), powdered and dissolved in water.

It was noticed, as pointed out by Marshall *et al* (1938) that M & B 693 is only partially soluble in water. As this rendered a true comparison with the other drug impracticable, in our next series we made an effort to administer M & B 693 in a soluble form. We decided to administer the drug after dissolving it in hydrochloric acid. The method adopted by us was as follows:—The required quantity of the drug was finely powdered and dissolved in a few drops of hydrochloric acid ; if this did not dissolve the drug completely, the solution was placed on a boiling water bath for a few minutes when the white milky suspension gradually changed into a clear solution. The pH was then adjusted approximately to that of the gastric juice with a few c.c. of a saturated solution of sodium bicarbonate and the solution was given to the animals with a copious amount of water.

The dose of M & B 693, that was given to the third group of animals by the above method, was 0.06 gm. per kg. or the same as that administered to the previous group.

To the last group of animals sulphanilamide in the form of Prontosil Soluble (manufactured by Bayer) was given in a dose of 0.09 g. per kg. by intramuscular injections into the gluteal muscles.

Methods of blood analyses:—

Throughout our present experiments we have estimated the total quantities of the drugs present in the blood. The method of analyses adopted was that of Marshall, Emerson and Cutting (11) as modified by Doble and Geiger (12). The reaction consists in the diazotization of the drug and coupling with diphenylamine. It was found by trial that the test

is equally applicable in case of both Prontosil Album and M & B 693. The yellow colour which was produced by diazotization was compared with standard solutions of sulphanilamide or sulphapyridine, as the case may have been, in a Pulfrich photometer, using filter No. S50 having a wave-length of 5300 A.U.

While estimating sulphanilamide following injections of Prontosil Soluble, it was noticed that the red colour of the substance was completely masking the yellow colour produced by diazotization. No other suitable method being available, we compared the colour of the alcoholic blood filtrate with standard solutions of Prontosil Soluble prepared in alcohol. The colour comparisons were made in a Pulfrich photometer, using filter No. S53 with a wave-length of 5300 A.U.

RESULTS

The blood concentrations observed after the oral administration of sulphanilamine and sulphapyridine are shown in Table I.

TABLE I
Showing the blood concentrations of sulphanilamide and sulphapyridine mg. per 100 c.c. following oral administration in monkeys.

| Animal.	Drug administd.	Dose g.m./kg.	Day of administration.	Blood concentrations before and after administration of drug.						
				Before	½ hr.	1½ hrs.	2½ hrs.	3½ hrs.	4½ hrs.	24 hrs.
No. 7	Prontosil Album (in water)	0.06	First	nil.	5.66	6.83	3.87	3.12	3.00	nil.
			Eighth	0.5	6.76	7.50	4.25	4.00	3.55	0.52
No. 8	do	0.09	First	nil.	4.59	6.45	—	5.62	—	nil.
			Eighth	1.35	7.08	8.76	—	5.62	—	—
No. 10	M & B 693 (in water)	0.06	First	nil.	0.71	1.78	—	2.67	—	nil.
			Fourth	1.12	—	2.37	—	3.04	1.46	nil.
No. 12	do	do	First	nil.	2.33	2.66	2.00	1.33	—	nil.
			Seventh	nil.	2.56	2.56	3.12	2.81	—	—
No. 11	M & B 693 (in HCl)	0.06	First	nil.	3.30	5.00	4.16	3.75	—	nil.
			Fifteenth	0.89	3.57	9.28	7.85	7.50	—	nil.
No. 13	do	do	First	nil.	3.00	5.00	2.35	2.06	—	—
			Seventh	nil.	3.12	7.62	4.70	4.70	—	—

There are quite a number of interesting points to be noted here. Following the first administration of sulphanilamide, the maximum blood concentrations reached in the two animals were 6.45 and 6.83 mg. per 100 c.c. respectively, while in cases where the sulphapyridine had been dissolved

in water and administered, the figures obtained were very much lower, being only 2.67 and 2.66 mg. per 100 c.c. But when sulphapyridine, dissolved in hydrochloric acid was given per os, the maximum concentrations in both animals were 5.0 mg. per 100 c.c., which is very near to the figures previously obtained with an identical dose of sulphanilamide.*

It can also be seen that following a dose of sulphanilamide, the maximum concentration was reached about an hour and a half after its administrations. Whereas in cases where sulphapyridine had been administered in an identical manner, this was arrived at about one to two hours later. However, when the sulphapyridine was dissolved in hydrochloric acid and administered, the rate of absorption was similar to that of sulphanilamide, the maximum concentration being reached only after an hour and a half.

The above points are clearly brought out in Fig. No. 1 which shows the blood concentration-time curves of animals No. 7, 10 and 11, each of which received identical doses (0.06 gm. per kg.) of sulphanilamide in water, sulphapyridine in water and sulphapyridine in hydrochloric acid, respectively.

FIG. 1

Time in hours.
A=animal No. 10, B=animal No. 11, C=animal No. 7
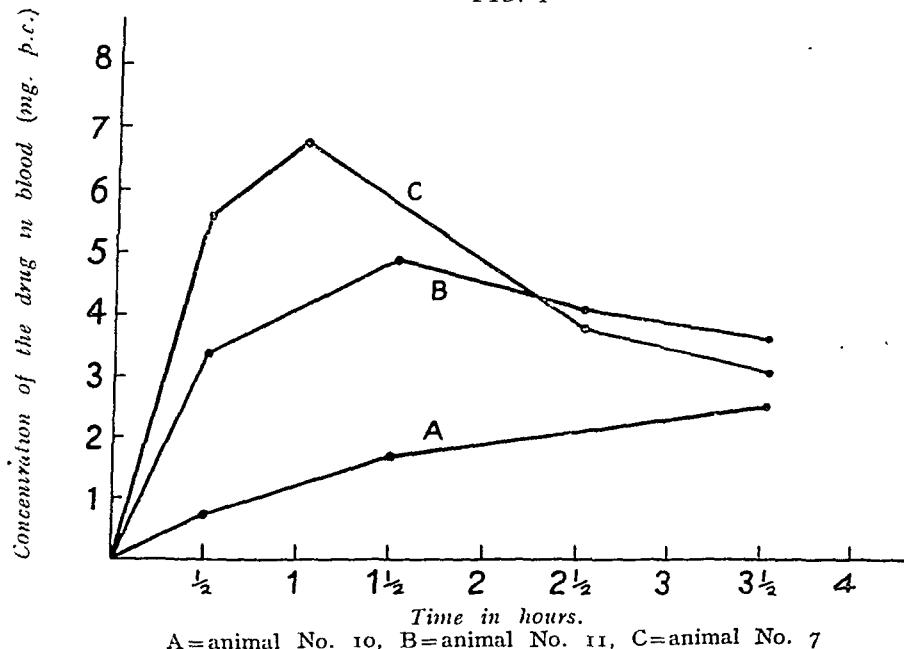
*A point to be noted here is that although animal No. 8 received a higher dose of sulphanilamide than No. 7, yet the maximum blood concentrations in the two cases were almost identical. As the animal receiving a higher dose is naturally expected to yield a higher figure than the one receiving a lower dose, this curious result would require an explanation. During the experiments this animal (No. 8) was found to develop diarrhœa, consequently the absorption of the drug in this case may not have taken place with as much facility as in case of a normal healthy animal, hence this discrepancy in the results.

The third important point to be noted from Table I is that in case of some of the animals (Nos. 7, 8, 10 and 11) when successive administrations of the drugs had already taken place over a number of days, the introduction of a dose caused a small quantity of the drug to persist in the blood even after twenty-four hours. It is also to be noted that the introduction of this fresh dose causes the blood concentration to rise to a level much higher than that which was recorded on the first day the drug was given. This is clearly shown in Fig. No. 2 in which are drawn the blood concentration-time curves of animals, Nos. 8, 10 and 11, as observed on the day the drug was given for the first time and again on a following day when the administrations had already taken place a number of times.

FIG. 2

Time in hours.
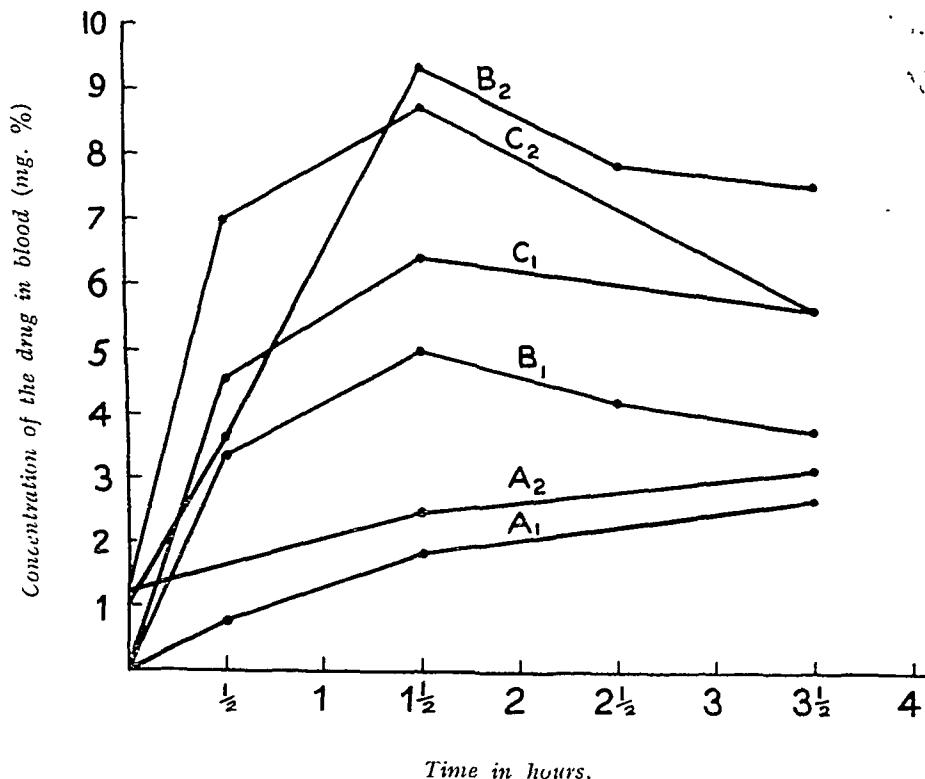
A_1 and A_2 = animal No. 10, B_1 and B_2 = animal No. 11, C_1 and C_2 = animal No. 8.

Table II shows the blood concentrations after intramuscular injections of sulphanilamide as Prontosil Soluble. The figures obtained here are several times higher than in case of those where the drug was given orally. The rates of absorption and elimination were also very much quicker, the maximum concentration being reached only after a half an hour and the drug was completely eliminated from the blood in two and a half hours.

TABLE II

Showing the blood concentrations of sulphanilamide in mg. per 100 c.c.
following intramuscular injections of the drug in monkeys.

| Animal. | Drug injected | Dose g./kg. | Blood concentrations | | | | | | |
			10 mts.	20 mts.	30 mts.	45 mts.	90 mts.	120 mts.	150 mts.
No. 4	Prontosil Soluble	0.09	27.3	28.5	31.5	21.3	7.0	1.67	nil
No. 14	do	do	—	—	24.25	—	7.0	—	nil

FIG. 3
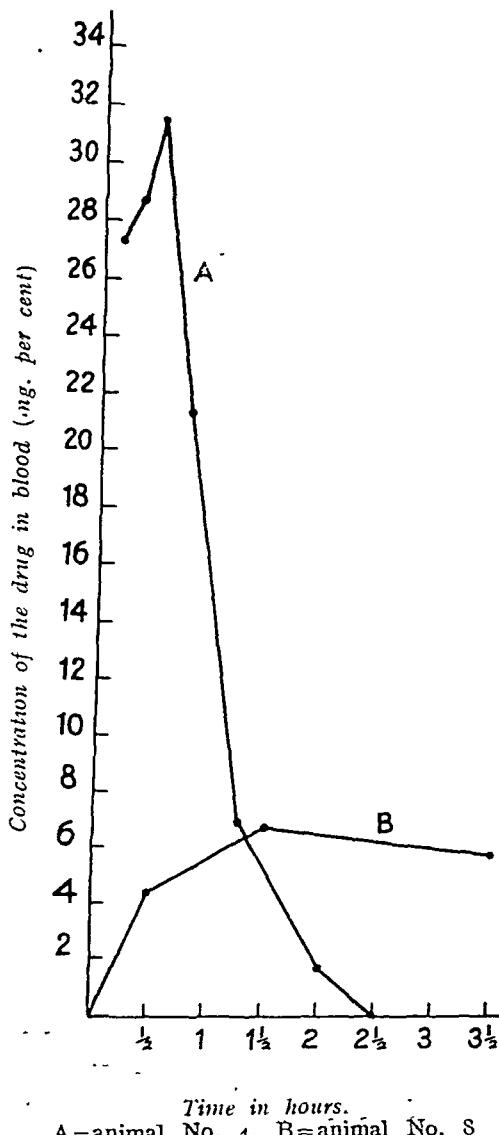
FIG. No. 3 demonstrates the striking difference in the blood concentration obtained by the two different methods of administration.

Time in hours.
A=animal No. 4, B=animal No. 8

DISCUSSION

The results of our experiments bring out several points which need clarification. One interesting observation is the difference in the relative rates and degrees of absorption of sulphanilamide and sulphapyridine when administered orally with water in an identical dosage. This is in accordance with the previous findings of Marshal *et al* (6, 7) and Powell and Chen (8) who attribute it to the very low solubility of sulphapyridine as compared with sulphanilamide. Wien (13) reported that this drug is less toxic than sulphanilamide, gram for gram. The former drug due to its low solubility and its consequent slow absorption might easily lead one to this conclusion. A drug cannot, however, be judged to be truly toxic or non-toxic by giving it to animals per os, without ascertaining the real nature of its passage into the blood. Highly toxic substances have some-

times been found to produce absolutely no toxic manifestations when introduced orally, because of poor absorption or destruction in the gastro-intestinal tract. Marshall and his associates (*loc. cit.*), working on the basis of blood concentrations, have come to the conclusion that sulphapyridine is more toxic than sulphanilamide, as observed from their relative lethal doses on mice, rabbits and dogs. This has also been found to be true by the writer from the point of view of their relative effects on the hæmopoietic system in monkeys (paper is under publication).

It was seen that when sulphapyridine was given orally in a soluble form, by dissolving it in hydrochloric acid, the absorption of the drug was very much improved. The blood concentration figures in such cases were about as high as in those where sulphanilamide was given per os. Marshall and his associates (*loc. cit.*) using a soluble sodium salt of sulphapyridine, have also found similar results and this has been later on confirmed by Powell and Chen (*loc. cit.*). But none of these recommend the use of this substance, as the buffering capacity of the gastric juice is insufficient to liberate the free acid completely from the salt, and moreover, the alkalinity of the substance is found to aggravate the discomfort of the patient due to nausea and vomiting. The alkaline reaction of the salt may also increase the toxicity of the drug. The method adopted by us, that of dissolving the drug in hydrochloric acid, was found to be quite satisfactory. No untoward symptoms were observed on the experimental animals, who seemed to tolerate the doses in a very satisfactory manner. This method of administration, therefore, seems to deserve a trial on human subjects. One may thereby introduce a smaller and more effective dose, instead of large and possibly wasteful amounts.

Now as regards the small quantities of the drugs being present in the blood, following a number of daily doses, it is quite likely that the repeated administrations bring about in the blood a state of equilibrium between absorption and excretion thereby causing certain amounts to be retained constantly in the circulation, provided the dose is repeated at regular intervals.

The phenomenon just now mentioned may partially account for the increase in the concentration levels, that become apparent when the animals have undergone treatment with repeated doses for some time. But the increase in most cases is so well marked that it cannot be due to this cause alone. Moreover, it can be seen, that even where (Nos. 12 and 13) no trace of the drug was present in the blood before the introduction of a dose, the concentration level was still found to mount up much higher than that recorded previously on the first day of the administration of the drug. It is quite likely that following repeated administrations, the animals got used to the drugs and absorption into the blood was thus facilitated, thereby causing the blood concentration levels to mount up higher with successive administrations.

The results of our experiments bring out another noteworthy point. This is the striking differences in the relative rates of absorption and elimina-

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tion, when the drugs are introduced by the two different routes, oral and parenteral. There was also a good deal of difference in the blood concentration levels in the two cases.

SUMMARY

A comparison has been made between sulphanilamide and sulphapyridine, as regards their blood concentrations following oral and parenteral administrations.

1. Sulphapyridine, when dissolved in water and given per os., was found to have a much lower blood concentration than sulphanilamide. The rate of absorption was also much slower.

2. When sulphapyridine was administered in a soluble form, being dissolved in hydrochloric acid, the rates and degrees of blood concentrations were almost identical with those obtained with sulphanilamide.

3. Sulphanilamide, when administered by intramuscular injections, was found to have a much quicker rate of absorption and elimination from the blood. The blood concentration level was found to be much higher in this case.

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PRESENCE OF B. COLI AGGLUTININ IN SERUM OF CHOLERA CASES AND THE POSSIBLE ROLE OF B. COLI IN CHOLERA

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(Received for Publication, December 10, 1940)

It has been observed that when sterile filtrates of cholera stools were injected into rabbits, considerable quantity of *B. Coli* agglutinin was found in their sera. It was, therefore, supposed that *B. Coli*, being the predominant micro-organisms inhabiting the lower intestinal tract, was likely to undergo rapid autolysis by the highly alkaline cholera stool. It is a well-known fact that autolysis of all bacteria is hastened in alkaline solution. These auto-lysed products of *B. Coli* might possibly be absorbed in the system along with the toxin of cholera from the intestine and might play an important rôle in producing or aggravating the symptoms of cholera. This work was, therefore, undertaken to ascertain whether agglutinin against *B. Coli* could be found in the serum of convalescent cholera patients.

EXPERIMENTAL

Blood and stool were collected from convalescent cholera patients, 7 to 15 days after the onset of the disease. *B. Coli* was isolated from the stool of the patients and agglutination test was made with patients' sera. Simultaneously agglutination test was also made with one Inaba strain of *Vib. cholera*. Owing to the variation of the antigenic character of different strains of *B. Coli* the use of a stock strain of the latter bacteria for agglutination test was abandoned. Agglutination tests were performed microscopically with serum diluted 1 in 20, 1 in 40 and 1 in 100. 35 Convalescent cholera cases, which showed in their sera positive agglutination with *Vib. Cholera (Inaba)*, were studied. The result is shown in the following tables. Controls from normal individual as well as from typhoid cases were also kept for comparative study.

TABLE I

Percentage of the presence of B. Coli agglutinin in sera of cholera patients.

Bacteria used.	No. of cases examined.	Positive case			Total No. positive cases.	Percentage of positive cases.
		1 in 20	1 in 40	1 in 100 and above		
Agglutinating *Vib. Cholera (Inaba)*	35		2	33	35	
Autogenous *B. Coli* from stool.	35	7 (20%)	9 (25.7%)	9 (25.7%)	25	71.4%

Table I shows that 71.4% of cholera patients develop agglutinin against autogenous *B. Coli*. For control, sera of normal individuals were also

tested for agglutination with autogenous *B. Coli*. Table II indicates the result of these cases.

TABLE II

Presence of B. Coli agglutinin in the sera of normal cases.

Bacteria used.	No. of cases examined.	Positive case			No. of positive cases	Percentage of positive cases.
		I in 20	I in 40	I in 100 and above		
B. Coli (autogenous)	50	8 (16%)	3 (6%)	3 (6%)	14	28%

Presuming that in other intestinal infection such as in typhoid fever similar absorption of autolysed *B. Coli* may take place, agglutination test was conducted with *B. Coli* isolated from stools of typhoid patients. The result is shown in Table III.

TABLE III

Number of cases of typhoid showing positive agglutination with autogenous B. Coli

Bacteria used	No. of cases examined.	Positive cases			Total No. positive cases.	Percentage of positive cases.
		I in 20	I in 40	I in 100 and above		
Autogenous B Coli	20	2 10%	2 10%	2 10%	6	30%

From the result shown in the above tables it is clear that the sera of convalescent cholera cases contained greater amount of *B. Coli* agglutinin both in number as well as in titre than in non-cholera or normal cases. It can be assumed, therefore, that a considerable amount of *B. Coli* was absorbed in the system of cholera patients through the intestine producing the titre of agglutinin as shown in the above tables.

From the light of the above experiments it appears to be very suggestive that *B. Coli* of the coecum and other parts of large intestine gets autolysed by the alkaline stool of cholera and this autolysed *B. Coli* after absorption may be responsible for the development or aggravation of certain symptoms in cholera, *e.g.*, vasomotor paralysis or toxæmia. The absorption of *B. Coli* antigen must have taken place during the acute stages of cholera because of the fact that at the latter part of cholera the intestine contains very few *B. Coli*. The abundant number of *B. Coli* in the lower intestine is autolysed rapidly by the highly alkaline stool during the first stage of cholera and it is presumed that this autolysed *B. Coli* is absorbed together with the toxin of cholera.

COMPLEMENT-FIXATION REACTION IN EXPERIMENTAL ANIMALS IN RESPONSE TO *PLASMODIUM KNOWLESI* ANTIGEN

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The observation that during the process of, and after the recovery from an infectious disease, the body acquired an immunity for a particular infection, received its first practical application in Jenner's vaccination against small-pox, a fact which was known long before the science of immunity came into existence. Scientific research on immunity is, however, of comparatively recent origin. It developed rapidly with the introduction of solid culture media in bacteriology. This provided pure cultures of bacteria for experimental purposes and gave an impetus to the study of various immunologic responses produced in the body when living and non-living agents of diseases gain a foothold in the tissues either under natural conditions or through parenteral administration.

Bacteriologists have taken full advantages of this natural immunological response of the body to bacterial infections and have produced vaccines and sera, which have played a highly important rôle in the prevention and cure of a wide variety of diseases and epidemics of bacterial origin. In fighting against protozoal diseases, however, this defence mechanism against protozoal infection has hardly yet been understood and utilised. One reason for our lack of information on this subject was the inability to get a sufficient amount of pure culture for experimentation. At present there is evidence of increasing interest in this subject. This similarity in the mechanism of antibody production and the conferring of immunity to bacterial and protozoal infections seems to be gaining credence even from erstwhile sceptics.

Success in the preparation of therapeutically efficient Leishmania vaccines (1) and the scrutiny of the existing literature concerning malarial epidemiological observations led us to consider the possibility also of malarial immunity. It has been observed in malaria-endemic areas that the spleen index diminished and immunity against malaria increased with increasing age. This indicated to us the probability of a gradual increasing immunity response occurring in a malarial infection. We were thus prompted to study the immunological aspects of the malaria problem and we have been engaged, now, for a number of years in experiments concerning the possibility of the production of malarial vaccines.

The importance of the investigation of this possibility is obvious, since à prophylactic and curative malaria vaccine would be a most-powerful weapon

in fighting malaria. Even if the immunization conferred is of short duration, the fact that malaria infection usually occurs during a relatively brief period in autumn, should make this temporary immunization of supreme value in controlling malaria. In fact, paradoxical though it might seem, even mosquito-bites of previously immunized persons are likely to enhance and maintain the immunity initially conferred. This constitutes, obviously, a fresh line of attack on the malaria problem, which is now being tackled by larvicidal and chemotherapeutic methods.

At present, the laboratory diagnosis of malaria rests on the findings of parasites in blood smears and one knows the difficulty associated with it in low grade infections and treated cases. The present investigation was carried out as a part of the problem comprising a comprehensive study of the nature of immunity and anti-body response in experimental animals which have been actively immunized with the variously extracted antigens of *Plasmodium knowlesi*. A suitable and potent malaria vaccine made of *Plasmodium knowlesi* antigen is being tried for its prophylactic and curative value in human malarial infections on groups of individuals under controlled conditions. The complement-fixation test as a specific serological method for the diagnosis of human malaria will form the subject of a subsequent paper.

Attempts have been made in the past by several workers to develop specific complement-fixation test for malaria but without much success. In our own series of experiments since 1935, we had been investigating the immunological response following the injection of cell-free *Plasmodium knowlesi* antigens in human beings and animals. A preliminary note of our work on complement-fixation test for malaria was recorded in the Annual Report for 1935-36 (2). The results of positive complement-fixation test, by using the antigen of *Plasmodium knowlesi* with the sera of 10 cases of human malaria with *Plasmodium vivax* infection was further recorded in the Annual Report for 1937-38 (3). Recently, Coggeshall and Eaton (4, 5) have made valuable contributions from the laboratories of the Rockefeller Foundation on the complement-fixation tests for malaria and we have been greatly benefited by their work in conducting our investigations. They obtained positive complement-fixation reaction by using the water-soluble antigens of *Plasmodium knowlesi* with the serum of monkeys infected with *Plasm. knowlesi*. It has been further reported by them that antigen prepared from blood and spleen of monkeys collected during acute phase of infection with *Plasm. knowlesi* acted as successful antigen for obtaining complement-fixation reactions with the sera of human beings infected with *Plasm. knowlesi*, *Plasm. vivax*, and *Plasm. falciparum*. Eaton and Coggeshall (6, 7) further demonstrated that the complement-fixing anti-bodies are produced in the serum of rabbits and monkeys in response to the injections of living or killed *Plasm. knowlesi* antigen.

MATERIALS FOR TESTS AND PROCEDURE

1. *Preparation of Antigen :*

Antigens used for our present series of complement-fixation tests were prepared from *Plasmodium knowlesi* extracted from infected red-cells and spleen of *Rhesus monkeys*.

A. EXTRACTION OF *Plasmodium knowlesi* ANTIGEN FROM PARASITIZED R.B.C.

So long as the problem of cultivation of malaria parasites remains unsolved, the best alternative is to obtain malaria antigen from culture *in-vivo*. *Rhesus* monkeys were infected with *Plasm. knowlesi* and bled from the heart when peripheral blood showed heaviest infection with mature parasites, about 50 to 60 per cent. The blood was defibrinated and centrifuged to separate the parasitized corpuscles, which form a grey layer between plasma and red-zone of non-infected cells.

Antigen 1 was prepared by physical method thus: Washed parasitized red-cells were suspended in sterile physiological saline solution in the proportion of 1 in 10 and hæmolysis of the cells was effected by alternate freezing and thawing for 3 to 4 times. It was then centrifuged and the parasitic deposit was washed and suspended in 0.5 per cent carbolised physiological saline solution. This was then kept in rubber-capped phials and preserved in refrigerator for future use.

Although in this method the red-cells were ruptured, the stroma did not get completely dissolved and fragments of it remained attached to the parasites leaving thereby chances of producing false reactions for the cell elements present. This method, however, has the advantage of being simple and providing lesser chance of microbic contamination.

Antigen 2 was prepared from parasitized R.B.C. by using the method of hæmolysin-cell-complement system: For production of anti-monkey R.B.C. hæmolysin various methods were tried. The method described here gave us the best result. A 10 per cent suspension of thoroughly washed monkeys' R.B.C. in physiological saline solution was made and 5 c.c. of it were injected into rabbits for five or six times twice weekly by intravenous route. If the titre was found satisfactory, the blood was collected from the heart of the rabbits. After separation of serum, the latter was put in a bath at 56° for half an hour to destroy complement and kept in rubber-capped phials for future use. It was found that the response in the production of anti-monkey hæmolysin following the injections of monkey R.B.C. in rabbits was usually much weaker than that against anti-sheep R.B.C. The titre of anti-monkey hæmolysin varied between 1 in 50 and 1 in 100. For effecting the hæmolysis of parasitized monkey R.B.C., requisite quantities of diluted amboceptor and guinea-pigs' complement were added to a 10 per cent suspension of parasitized cells and the mixture was placed in an

incubator for half an hour. The parasites freed from cells were then centrifuged and washed in normal saline. The number of parasites per c.c. of the suspension was counted in hæmocytometer after making suitable dilutions in saline containing Brilliant Cresol Blue. The parasites were then re-suspended in 0.5 per cent carbolised saline and kept in refrigerator for future use.

Antigens prepared by this method were found to be almost free from blood elements and the parasites appeared to be least damaged.

Antigen 1 and Antigen 2 formed the immunizing antigens for monkeys and rabbits.

Antigen 3 was prepared by physical method by subjecting washed parasitized red-cells to alternate freezing and thawing over a period of 4 to 5 days with addition of distilled water or saline in the proportion of 1 in 10. After repeating the process for several times when hæmolysis of the cells was complete, it was centrifuged and the supernatant fluid was put in ampoules and kept in refrigerator for future use.

Antigen 3A, which forms the control antigen, was prepared by the method given above but washed red-cells from *non-infected* monkeys were used instead of those from the infected monkeys.

Antigen 4 was prepared from infected defibrinated whole blood collected at the height of infection. It was subjected to repeated freezing and thawing and when completely hæmolysed centrifuged and the supernatant fluid was used as antigen.

Antigen 5 consisted of plasma *alone* of the infected whole blood collected at the height of infection.

Antigen 6 was obtained by the alcoholic method of extraction of the antigen from the parasitized cells similar to that used for extracting Bordet-Ruelens antigen in Wassermann test.

Antigen 7 was prepared from the parasitized cells by combined ether and alcohol extraction as is used in preparing Kahn antigen.

Russell's viper venom and cobra venom hæmolysins are being tried on parasitized cells for the preparation of potent *Plasmodium knowlesi* antigen. Cobra venom hæmolysin when allowed to act on lecithin, produces a hæmolytic substance which has got a very strong hæmolytic action on R.B.C.

Further, chemical methods of hæmolysis of R.B.C., *e.g.*, with taurocholate, saponin etc., which were tried in our earlier tests, were not used in the present series of experiments.

B. EXTRACTION OF *Plasmodium knowlesi* ANTIGEN FROM INFECTED SPLEEN OF MONKEYS

Antigen 8 was extracted from the infected spleen of monkeys at the terminal phase of acute infection. The spleen was cut into small pieces and dried in vacuum desiccator at room temperature. The dried substance

was then powdered and 5 c.c. of distilled water were added to each gram of spleen powder. It was then subjected to repeated freezing and thawing over a period of 4 to 6 days and then put on a shaking apparatus overnight. The product was then centrifuged and the supernatant fluid was removed, and diluted with an equal volume of 1.7 per cent saline solution. This was put in ampoules and kept in refrigerator.

Antigen 8A, which forms the control antigen, was prepared in the same manner as Antigen 8, but spleens of *non-infected* monkeys were used instead of those from infected monkeys.

Antigen 9 consisted of alcoholic method of extraction of infected spleenic substance.

Antigen 10 consisted of combined ether and alcohol extraction of infected spleenic substance.

0.5 Per cent phenol used as preservative for the antigens, was found to produce certain amount of auto-agglutination of the parasites but otherwise proved satisfactory. One lot of such antigen preserved for over four years in ice chest was found to be antigenic. Formalin, when used for the same purpose, did not produce any appreciable auto-agglutination, but was probably responsible for the loss of potency of some of the antigens.

Antigens 3 to 10 formed the complement-fixing antigens under our present investigation.

2. *Preparation of Immune Sera :*

Rabbits and monkeys were actively immunized with the Antigens 1 and 2, extracted by methods of freezing and thawing and hæmolysin-cell-complement system. The injections were given bi-weekly in gradually increasing doses of the antigens by intravenous route. Rabbits received usually six injections of 25, 50, 100, 150, 200 and 250 million parasites while monkeys required bigger doses, 250, 500, 750, 1000, 1500 and 2000 million parasites to enable them to give rise to antibodies in detectable quantities.

Antibody titration was made before the immunization was started and in most of the cases weekly estimations of the antibodies were made from the beginning of immunization till the reaction became negative after the immunization had been stopped. In a few cases a single injection was made after the antibodies had disappeared from the blood and the effect noted by titration of their sera.

Monkeys and rabbits after immunizations were bled and their sera inactivated in a bath at 56°C for half an hour.

3. *Complement*—fresh sera from guinea-pigs.

4. *Amboceptor*—specific immune hæmolysin for sheep R.B.C.

5. *Sheep Cells*—5 per cent. suspension of washed R.B.C. of sheep.

6. *Physiological saline solution*—(0.85 per cent.).

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PROCEDURE

Titration of Amboceptor: Amboceptor was daily titrated keeping the quantity of complement and R. B. C. constant. 0.1 C.c. of different dilutions of amboceptor was used with 0.1 c.c. of complement (1 in 2) and 0.4 c.c. of 5 per cent sheep R.B.C. emulsion. The total volume was made up to 2 c.c. with addition of normal saline solution and incubated at 37°C for 15 minutes, after which reading was taken. The maximum dilution of amboceptor which caused complete hæmolysis was taken to be the working titre.

SCHEME 1.

Showing the procedure of titration of Amboceptor

Test tube No.	Sheep cells 5 p.c.	Comple- ment. Dil. 1 in 2	Saline 0.85 p.c.	Amboceptor			Result.
				Dilution	Amount		
1	0.4 c.c.	0.1 c.c.	1.4 c.c.	1 in 100	0.1 c.c.	Incubated for 15 minutes at 37°c.	Complete hæmolysis.
2	0.4 c.c.	0.1 c.c.	1.4 c.c.	1 in 150	0.1 c.c.		Complete hæmolysis.
3	0.4 c.c.	0.1 c.c.	1.4 c.c.	1 in 200	0.1 c.c.		No hæmolysis.

So here the working titre of the amboceptor is 0.1 c.c. of 1 in 150 dilution. In our work, dilution of amboceptor varied from 1 in 130 to 1 in 160. In antigen titration and also in actual test, this amount of amboceptor was used with 0.2 c.c. instead of 0.4 c.c. of sheep cells in order to make an allowance for the anti-complementary properties of antigen and serum.

Titration of Antigen: After preparation of antigens, these were tested for their anti-complementary and hæmolytic properties as shown below in Scheme 2. Although the alcoholic extracts of antigen were usually anti-complementary up to the dilution of 1 in 16, the saline extracts were anti-complementary only when used undiluted or diluted, 1 in 2. The saline extracts were usually not hæmolytic. In actual test the antigen was diluted four times the minimum dilution which was found to be not anti-complementary during antigen titration. For example, in the case of antigen 5B (Scheme 2) 0.1 c.c. of undiluted antigen was completely anti-complementary, 0.1 c.c. of 1 in 2 dilution was partially anti-complementary and 0.1 c.c. of 1 in 4 had no anti-complementary properties. So in actual test 0.1 c.c. of dilution 1 in 16 of this antigen was used. In case of antigen N.B. 1, it was observed that 0.2 c.c. of undiluted antigen was slightly anti-complementary, whereas 0.1 c.c. was not anti-complementary. So in actual test 0.1 c.c. of 1 in 4 dilution of this antigen was used. The sensitiveness of antigens prepared by different methods was determined by finding the maximum dilution of

the antigen which would give complement-fixation test with a single positive serum or by finding the maximum workable strength of antigen against various dilutions of the positive serum.

SCHEME 2.

Showing the procedure of titration of Antigen

Test tube No.	Antigen Lot. 5B.	Complement. Dil. 1 in 2.	Saline 0.85 p.c.		Sheep R.B.C. emulsion 5%	Amboceptor titrated.		Results.*
1	0.1 c.c. undiluted	0.1 c.c.	1.5		0.2 c.c.	0.1 c.c.		No hæmolysis.
2	0.1 c.c. 1 in 2	0.1 c.c.	1.5	Incubated for 1 hr. at 37°.	0.2 c.c.	0.1 c.c.	Incubated at 37°.	Partial hæmolysis.
3	0.1 c.c. 1 in 4	0.1 c.c.	1.5		0.2 c.c.	0.1 c.c.		Complete hæmolysis.
4	0.1 c.c. undiluted	0.1 c.c.	1.6		0.2 c.c.	—		No hæmolysis.
5	—	0.1 c.c.	1.6		0.2 c.c.	0.1 c.c.		Complete hæmolysis.

*Result was read when the complete hæmolysis was obtained in tube No. 5.

Adsorption of sera was usually performed with monkey R.B.C., when the sera gave false positive reaction with normal blood antigen. But as reported by Coggeshall and Eaton, by such adsorption, the sera often became anti-complementary. The sera of monkeys and rabbits proved to be more anti-complementary than human sera even when blood was collected in the morning in fasting condition. This was more marked in sera of monkeys collected during acute and sub-acute conditions.

SCHEME 3.

Showing the procedure of complement-fixation test.

Tubes	Test Series	Serum diluted or un-diluted.	Complement Dil. 1 in 2	Antigen titrated	Saline 0.85%		Sheep cells 5%	Amboceptor titrated	
1	Unknown serum.	0.2 c.c.	0.1 c.c.	0.1 c.c.	1.3 c.c.		0.2 c.c.	0.1 c.c.	
2	Serum control	0.2 c.c.	0.1 c.c.	—	1.4 c.c.	Incubated for 1 hr. at 37°c.	0.2 c.c.	0.1 c.c.	Incubated at 37° and reading of tube No. 1 was taken when there was complete hæmolysis in tubes 2, 3 and 4.
3	Antigen control	—	0.1 c.c.	0.1 c.c.	1.5 c.c.		0.2 c.c.	0.1 c.c	
4	Hæmolytic control	—	0.1 c.c.	—	1.6 c.c.		0.2 c.c.	0.1 c.c	
5	Saline control	—	0.1 c.c.	—	1.7 c.c.		0.2 c.c.	—	

. Tube No. 2 was taken to ascertain if the serum was anti-complementary, and tubes No. 3 and 4 served as antigen and hæmolytic control. Tube No. 5 was set up as saline control. Result was noted as 3 + positive where there was no hæmolysis, 2 + positive where slight hæmolysis, 1 + positive where fair hæmolysis and – (negative) where complete hæmolysis was observed in tube No. 1.

Comparative studies of antigens from the spleen, blood and plasma of the same monkey with acute infection due to *P. knowlesi* as tested against a known positive and a known negative serum are given below.

TABLE I.

Showing the comparative value of different antigens

Monkey No.	Antigen Lot No.	Antigen extracted from.	Mode of extraction.	Antigen dilution.	Complement-fixation reaction with one negative serum.	Complement-fixation with one positive serum.
M. 10 heavily infected.	4A	Parasitized R.B.C.	Freezing and thawing in distilled water.	1 : 4	Neg.	+ + +
	4B	Parasitized R.B.C.	Freezing and thawing in saline.	1 : 2	Neg.	+ + +
	4C	Infected spleen	Freezing and thawing in saline.	1 : 8	Neg.	+ +
	4D	Plasma from acute infection		1 : 8	Neg.	Neg.
M. 9 heavily infected.	5A	Parasitized R.B.C.	Freezing and thawing in distilled water.	1 : 16	Neg.	+ + +
	5B	Parasitized R.B.C.	Freezing and thawing in saline.	1 : 16	Neg.	+ + +
	5C	Infected spleen	Freezing and thawing in distilled water.	1 : 8	Neg.	+ + +
	5D	Infected spleen	Freezing and thawing in saline.	1 : 6	Neg.	+ + +
	5E	Normal R.B.C.	Freezing and thawing. in saline.	1 : 4	Neg.	+ .
M. 3 heavily infected.	P1	Plasma + Parasitized R.B.C.	Freezing and thawing.	1 : 20	Neg.	+ + +
	P2	Plasma from acute infection	Pure.	1 : 4	Neg.	Neg.
	6A	Infected spleen	Freezing and thawing in saline.	1 : 8	Neg.	+ + +

Monkey No.	Antigen Lot No.	Antigen extracted from.	Mode of extraction.	Antigen dilution.	Complement-fixation reaction with one negative serum.	Complement-fixation with one positive serum.
M. 17 normal.	N.B. 1	Non-infected R.B.C.	Freezing and thawing in saline.	1 : 4	Neg.	+
	N.B. 2	,,	Freezing and thawing in distilled water.	1 : 4	Neg.	+
	N.S. 1	Non-infected spleen.	Freezing and thawing in saline.	1 : 4	Neg.	Neg.
	N.S. 2	,,	Freezing and thawing in distilled water.	1 : 8	Neg.	Neg.
	P3	Non-infected monkey's plasma.	Pure.	1 : 4	Neg.	Neg.
M. 6-7 heavily infected.	A1	Parasitized R.B.C.	Alcoholic extraction.	1 : 20	Neg.	Neg.
	AE1	,,	Combined Ether-Alcohol extract.	1 : 20	Neg.	Neg.
	A2	Infected spleen.	Alcoholic extraction.	1 : 30	Neg.	Neg.
	AE2	,,	Combined Ether-Alcohol.	1 : 30	Neg.	Neg.

It will be seen from Table I, that while distilled water and saline extracts of both spleen and infected blood of monkeys at the height of infection with *P. knowlesi* gave satisfactory complement-fixation reaction, alcoholic extracts failed in that respect. Plasma of monkeys at the height of acute infection did not produce complement-fixation to any degree of satisfaction as claimed by Eaton (8).

Fixation of complement in comparatively low positive reaction with the positive sera by non-infected blood antigen N.B. 1 and 2 may possibly be due to the presence of antibodies against monkey red corpuscles in the positive serum as a result of injections of traces of stroma of red cells present in the immunizing antigen. The weakly positive reaction with 5E antigen may also be explained in a similar manner.

TABLE II.

Comparative sensitiveness of blood and spleen antigens.

Serum.	Dilution.	Antigen from infected blood Lot. 5B.	Antigen from non-infected blood Lot. N.B. 1.	Antigen from infected spleen Lot. 4C.	Antigen from non-infected spleen Lot. N.S. 1.
R.113.D	1 in 2	+ + +	Neg.	+ + +	Neg.
	1 in 4	+ + +	Neg.	+	Neg.
	1 in 8	+ + +	Neg.	Neg.	Neg.
	1 in 16	+ +	Neg.	Neg.	Neg.
R.113.C	1 in 2	+ + +	+	+ +	Neg.
	1 in 4	+ + +	+	+ +	Neg.
	1 in 8	+	Neg.	Neg.	Neg.

It will be seen in Table II that of the saline extracts, the antigens prepared from parasitized blood appeared to be more sensitive than those prepared from the spleen of infected monkeys. The comparative sensitiveness of the two lots of antigens prepared either from blood or from spleen probably depended on the amount of parasitic substance per volume of the sample of blood or spleen. For it will be seen in Table I that while the antigen from the spleen of monkey M. 10 gave moderately positive reaction with the positive serum, the spleenic extract of monkey M. 9 gave strongly positive result with the same serum although both the antigens were prepared in the same way and the peripheral blood of both showed almost similar parasitic count at the time of bleeding. It has also been observed that spleenic antigen prepared from particular monkey, M3, with highest parasite count before bleeding to death showed the same and occasionally even a greater degree of sensitiveness than blood antigens from other monkeys. It may also be remembered that while by centrifuging one can concentrate the parasitized R.B.C., there is no method of concentrating spleenic material harbouring *P. knowlesi*.

Eighteen rabbits were immunized by injections of *P. knowlesi* antigen prepared in different ways as described before. It has been found that all these antigens were capable of producing complement-fixing antibodies, when injected into rabbits. Each rabbit received between 2 and 6 injections. Although individual variations were noted in the rabbits regarding the time required for the appearance of antibodies in detectable quantities in their sera after the injections of the antigen, the height of titre reached and the duration of circulating antibodies in the blood after immunization had been stopped, the following facts may be taken as an average.

After the injection of a moderate dose of antigen in a rabbit, complement-fixing antibodies could be detected in its serum in 8—10 days' time and the maximum titre is usually reached between 2 and 3 weeks. The highest

titre of complement-fixation reaction commonly attained was between 1 in 8 and 1 in 16 dilutions of the serum. This titre is maintained with minor fluctuations for 2—4 weeks, after which the titre began to fall, with minor degrees of fluctuations from time to time. Complement-fixation reactions usually became negative in another 4—8 weeks' time. When the reaction of sera had become negative, a comparatively small dose of antigen would again bring the reaction of the sera to a high positive titre.

TABLE III.

Complement-fixation reaction before and after immunization.

Experimental animal.	Immunized or not.	Sera from No. of rabbits tested.	Reaction with antigen from infected monkey (blood or spleen).					Reaction with antigen from normal monkey (blood or spleen).				
			No. of tests.	3 +	2 +	1 +	Neg.	No. of tests.	3 +	2 +	1 +	Neg.
A. Rabbits	Before immunization.	18	36	0	2	2	32	36	0	2	3	31
	After immunization.	17*	207	124	38	22	23	207	12	24	44	127
B. Monkeys	Before immunization.	6	12	0	0	0	12	12	0	0	0	12
	After immunization.	6	24	2	6	8	8	24	0	2	2	20

*One died in process of immunization.

It would be seen from Table III that of the 18 rabbits' sera, each tested with antigens from infected spleen and blood in a total number of 36 tests, 32 or 88.8% gave negative complement-fixation reaction, and with antigens from normal spleen and blood, 31 or 86.1% gave negative results. After immunizations have started in the same group of rabbits, one animal died and of the remaining 17 rabbits samples of their sera were tested 207 times against antigens prepared from spleen and blood of infected as well as from normal monkeys. With the antigen from infected spleen or blood, 23 tests or 11.1% were negative while 124 or 59.9% gave strongly positive, 38 or 18.3% moderately positive and 22 or 10.6% weakly positive reactions. With antigen from normal blood cells and spleen 127 tests or 61.3% were negative, 12 or 5.7% were strongly positive, 24 or 11.5% were moderately positive and 44 or 21.2% weakly positive.

It will be seen that while false positive reactions were comparatively few with non-immunized animals, they were comparatively frequent with immunized rabbits' sera. This was due to the presence of traces of stroma of R.B.C. in the immunizing antigen, while none of the antigens for the complement-fixation tests was free from the blood elements. This fact is demonstrated in Table IV.

It may be noticed in Table III that antibody response in monkeys were comparatively weak. It was also observed that in monkeys complement-fixing antibodies appeared in the sera comparatively late, the height of titre remained low and disappeared more quickly when compared with those of the rabbits. It also required much bigger doses and larger number of injections for the appearance of complement-fixing antibodies in the sera of monkeys. False positive reactions with immunized monkeys' sera were comparatively very few.

TABLE IV.

Complement-fixation reaction with antigen prepared from parasitised and normal blood.

Serum of rabbits.	Rabbit No.	Sera diluted or undiluted.	Reaction with antigen from parasitised blood.	Reaction with antigen with blood from non-infected monkey.
1. Normal.	16	undiluted	—	—
2 Immunized by normal monkey R.B.C.	H.1	(a) undiluted	+ + +	+ + +
		(b) 1 in 2	+ + +	+ + +
		(c) 1 in 4	+ + +	+ + +
		(d) 1 in 8	+ + +	+ + +
		(e) 1 in 16	+ + +	+ + +
3. Immunized by stroma of normal monkey.	98	(a) undiluted	+ + +	+ + +
		(b) 1 in 2	+ + +	+ + +
		(c) 1 in 4	+ + +	+ + +
		(d) 1 in 8	+ + +	+ + +
		(e) 1 in 16	+ + +	+ + +
4. Immunized by parasitised R.B.C.	113C	(a) undiluted	+ + +	+ + +
		(b) 1 in 2	+ + +	+ +
		(c) 1 in 4	+ + +	+
		(d) 1 in 8	+	—
		(e) 1 in 16	—	—
5. Immunized by malaria vaccine —parasitic substance obtained by hæmolysin-cell-complement system.	138A	(a) undiluted	+ + +	+
		(b) 1 in 2	+ + +	—
		(c) 1 in 4	+ + +	—
		(d) 1 in 8	+ +	—
		(e) 1 in 16	+	—

It was seen that antigens prepared from monkeys' spleen gave false positive reactions less often than antigens prepared from the blood. In cases of false positive reaction with blood antigens, complement-fixation test with antigens from spleen gave better indications of the result. But as stated before blood antigens were usually more sensitive than spleenic antigens.

TABLE V.

Hæmolytic titre of different rabbits' sera against monkey R.B.C.

Sera of rabbits.	Rabbit No.	Dilution of serum.				
		undiluted	1 in 2	1 in 4	1 in 8	1 in 16
1. Normal.	16	—	—	—	—	—
2. Immunized by normal monkey R.B.C.	H.1	+ + +	+ + +	+ + +	+ + +	+ + +
3. Immunized by stroma of normal monkey R.B.C.	98	+ + +	+ + +	+ + +	+ + +	+ + +
4. Immunized by parasitized R.B.C.	113C	+ +	+	+	—	—
5. Immunized by malaria vaccine.	138A	—	—	—	—	—

On comparing the results tabulated in Tables IV and V, it will be seen that complement-fixation reaction of different rabbits' sera with normal monkey blood antigen roughly corresponds with their hæmolytic titre against monkey R.B.C. Whereas both the tests are practically negative with 1st and 5th groups of sera, they are strongly positive with 2nd and 3rd group. The reactions are weaker with 4th group sera. This is due to the fact that the the blood element present in the immunizing antigen was less in group 4 than in groups 2 and 3. It would be noticed that sera of the 5th group rabbits immunized with malaria vaccine showed no hæmolysin against monkey R.B.C. and very little complement-fixing antibodies with antigen from normal monkey R.B.C.

Attempts were made to adsorb the sera showing false positive complement-fixing reaction with normal blood antigens from red blood cells of non-infected monkey. Results obtained were unsatisfactory. Because many sera after such absorption became anti-complimentary. Of the remaining sera, the effect of adsorption was incomplete in most cases. These sera showed lower titre of complement-fixation but only in rare occasion the false positive sera could be converted into totally negative ones with normal antigens by adsorption with blood of non-infected monkeys. With some of the sera the effect of such adsorption was totally negative.

DISCUSSION

The malaria vaccines used for actively immunizing rabbits and monkeys consisted mainly of parasitic substance of *P. knowlesi* but they also included

traces of stroma of red blood cells and possibly small quantities of other non-specific elements such as leucocytes and plasma.   The antibodies in response to these vaccines in the sera of laboratory animals were therefore multiple.   The antigen for complement-fixation test was also of a complex nature.   The results of complement-fixation tests with the   immune sera had to be considered with certain reserves and   therefore various control experiments were conducted to eliminate the possible sources of error in estimating the values of the complement-fixation test.   But taking all the above facts into consideration, there remains no difficulty in understanding the correct interpretation of the results.

It would be seen from Table I that antigen for complement-fixation reaction with sera of rabbits and monkeys immunized by malaria vaccine prepared with *P. knowlesi* is best obtained by saline extracts from spleen and blood of monkeys at the terminal phase of acute infection with *P. knowlesi*.   Alcoholic extracts were practically useless.   Of the antigens prepared from blood and spleen, those prepared from blood were more sensitive than those prepared from spleen.   But antigens from blood were more liable to give false reactions than those from spleen.   This was again due to the traces of blood elements in the vaccines used for immunizing rabbits.   Plasma from monkeys with acute infection did not serve as useful antigen for complement-fixation test in this series of tests.

Rabbits although non-susceptible to *P. knowlesi* infection when artificially immunized with *P. knowlesi* vaccine, gave much better complement-fixation reaction in their sera, than the naturally susceptible *Rhesus* monkeys under similar circumstances.   Thus all the 17 rabbits immunized either with the vaccines or with parasitized red blood cells of monkeys showed fairly high titre of complement-fixing antibodies in their sera in about two weeks' time. The titre naturally varied from rabbit to rabbit depending on the quantity of the immunizing antigens.   Marked individual variation among different animals were also noticed when all other things were kept constant.

False positive reaction with normal blood antigen and hæmolytic titre against monkey R.B.C. ran parallel as in Tables IV and V.   Whether the antibodies concerned in both these reactions belong to one or the same group was not thoroughly investigated in the present series of experiments.   But it appeared that all sera giving false positive reactions were not hæmolytic to monkey R.B.C.

SUMMARY

1.   *Plasmodium knowlesi* vaccine free from R.B.C., when injected into rabbits and monkeys, gave rise to complement-fixing antibodies in their sera.

2.   Suitable complement-fixing antigens were prepared from water soluble extracts from the spleen and blood of monkeys during their terminal phases of acute infection with *P. knowlesi*.

In conclusion we offer our grateful thanks to the Government of Bengal for a grant to enable us to carry out a comprehensive investigation on problems connected with immunity in malaria.

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A NOTE ON THE AGGLUTINATING TITRE OBTAINED IN A RABBIT AGAINST A STRAIN OF *LEISHMANIA TROPICA* AFTER ACTIVE IMMUNIZATION

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Before producing active immunization, the serum of a rabbit was tested for normal agglutinin, which was found to be 1:20.

A strain of *Leishmania tropica* was selected and an homogenous emulsion of the same was made and adjusted to Brown's opacity tube No. 10.

Progressively higher doses of the emulsion, starting from 1 c.c. to 5 c.c., were injected intravenously every fourth day, the total number of injections being five. Four days after each injection and finally on the 8th and 10th days after the fifth, blood was taken directly from the heart and the serum after being separated was tested for agglutination with the specific antigen.

After the 1st, 2nd, 3rd and 4th injections, the titre was respectively 1:80, 1:640, 1:5,000 and 1:10,000.

The highest titre obtained after the last injection rose up to 1:20,000.

A reference may be made to the previous record of rise of titre, obtained by Noguchi (1) being 1:100 and later by Ray (2) being 1:2,500.

My thanks are due to Dr. J. C. Ray for his kind interest and advice.

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STUDIES ON THE MECHANISM OF VEGETABLE TISSUE RESPIRATION. PART I. FUMARASE AND SUCCINO-DEHYDROGENASE

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Polyphenoloxidase and peroxidase have long been known to be widely distributed in vegetable tissues and it was generally believed that these two enzymes were chiefly concerned with the respiration of vegetable tissues. The researches of Palladin, Bach, Chodat, Robinson, Raper, Onslow and Szent-Györgyi are mostly responsible for the present knowledge of the mechanism of these two enzymic reactions and their rôle in respiration of vegetable tissues.

Szent-Györgyi (1) is of opinion that polyphenol-oxidase does not play any part in the respiration of vegetable tissues, but might have something to do with natural immunity and the protection of the plant against damage. He made exhaustive studies in order to find out the respiratory system in which peroxidase might be involved and came to discover ascorbic acid which is present in relatively large quantities in the so-called "peroxidase plants". He also found in vegetable an enzyme, ascorbic acid oxidase. He has recently formulated a theory for the respiration of vegetable tissues. Molecular oxygen activated by ascorbic acid oxidase oxidises ascorbic acid whereby dehydroascorbic acid and hydrogen peroxide are formed. Hydrogen peroxide interacting with peroxidase oxidises a flavone. The oxidised flavone again oxidises a second molecule of ascorbic acid. Dehydro-ascorbic acid is finally reduced by enzymic systems present in tissues. In this way respiration of vegetable tissues continues by alternate oxidation and reduction of ascorbic acid.

But reduced cozymase (diphosphopyridine nucleotide) does not reduce dehydroascorbic acid, so it is still left for investigation which system reduces dehydroascorbic acid in the tissues. It has been reported that dehydro-genases acting on glucose (Mann, 2) hexosemonophosphate (Meldrum and Tarr, 3) and citric acid (Banga, 4) reduce glutathione and reduced gluta-thione reduces dehydroascorbic acid. Hopkins and Morgan (5) found that this reduction is greatly accelerated by ascorbic acid oxidase. But this

fact could not be confirmed by Kertesz (6). Crook and Hopkins (7) later attributed this failure of Kertesz to the lability of the enzymic system. All these facts clearly present various complications in the mechanism but at the same time indicate a chain of reactions linking up the molecular oxygen with the "donator of hydrogen" or the "foodstuff".

Recent discoveries of "diaphorase" (Adler and *et al.*, 8) or the "co-enzyme factor" (Dewan and Green, 9) which is the malonate-insensitive enzyme reducing cytochrome in presence of reduced coenzymes (di- and tri-phosphopyridine nucleotides), and of cytochrome oxidase (Bhagvat and Hill, 10) *the enzyme oxidising reduced cytochrome in vegetable tissues*, have still more complicated the problem of the mechanism of respiration in vegetable tissues.

The present investigation has ·been undertaken with the object of throwing light on the rôle of cytochrome and C_4-dicarboxylic acids in the respiration of vegetable tissues.

C_4-Dicarboxylic acids like succinic, fumaric, malic and oxalacetic acids have been found to play very fundamental rôles in the respiration of animal tissues. They catalytically promote the oxidation of carbohydrates in presence of tissues (Szent-Györgyi, 1 ; Das, 11). The activated hydrogen of the "foodstuff" or the metabolite is taken up by oxalacetic acid present in the tissue to form malic acid by malic dehydrogenase. This malic acid is converted into fumaric acid by a specific enzyme, fumarase ; and fumaric acid is ultimately reduced to succinic acid by succino-dehydrogenase. Hydrogen from malic acid is carried over to fumaric acid by the yellow-enzyme (Szent-Györgyi, 1). Succinic acid in its turn donates the hydrogen to cytochrome forming reduced cytochrome. The reduced cytochrome is oxidised by molecular oxygen activated by "Atmungsferment" or cyto-chrome oxidase. We thus find that C_4-dicarboxylic acids form a bridge between the activated hydrogen of "foodstuff" and the activated molecular oxygen, over which hydrogen travels by steps and ultimately combines with oxygen through the intermediary of cytochrome whose ferrous ion is oxidised to the ferric state.
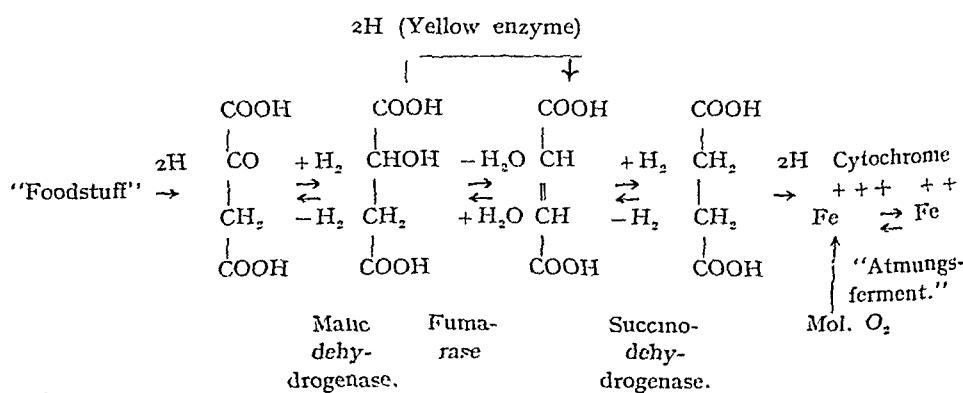
The wide occurrence of all these organic acids in vegetable tissues presents many interesting suggestions. Much has been done on the metabolism of these organic acids in vegetable tissues and studies have been made regarding the mechanism of reactions involved in their formation and loss in vegetable tissues and also enquiries into the rôle which these reactions play in the life of the plant. But no definite conclusion can yet be formed till more work has been done in this direction. Undoubtedly this is a most fascinating field of enquiry to a plant biochemist.

Numerous investigations have been carried out on the qualitative and quantitative estimation of organic acids in plant materials but the enzymes responsible for metabolism of these acids have not been much studied. In this preliminary paper studies on two of the most interesting enzymes, namely fumarase and succino-dehydrogenase, have been reported and the bearing of these enzymes on the mechanism of vegetable tissue respiration and the formation of asparagine has been discussed.

EXPERIMENTAL

Method.—Fumarase activity was determined by estimating the quantity of *l*-malic acid formed from fumaric acid at different intervals. In all the experiments the reactions were allowed to take place with the same quantity of sodium fumarate in equal volumes of reaction mixtures under identical conditions. Definite and equal quantity of the reaction mixture was pipetted out and deproteinised with equal volumes of sulphuric acid (10%) and sodium tungstate (10%) at different intervals.

l-Malic acid was estimated in the deproteinised solution as uranium salt according to the method of Straub (12). The deproteinised solution was brought to pH 6.0., warmed and saturated with uranium acetate. It was then cooled in ice, filtered and *l*-malic acid was estimated polarimetrically in a 20 cm. tube.

Succino-dehydrogenase activity was determined according to Thunberg technique using 2 : 6-dichlorophenolindophenol as hydrogen acceptor.

Plant tissue respiration was measured manometrically in Warburg's apparatus at 23°, using slices of germinated pea as plant material. The slices were made with very sharp razors and great care was taken to make the slices of uniform thickness. Known quantities of these sliced tissues were introduced into Warburg's vessels and was finally suspended in nutrient solution (pH 6.5). Alkali was introduced into the central chamber in order to absorb carbon dioxide produced during respiration and the volume of oxygen uptake was noted.

Seeds.—All the seeds were procured from Sutton and Sons, Calcutta. Experiments described in this paper were done with pea unless mentioned otherwise. Barley and rice were from Imperial Agricultural Research Institute, Pusa.

Nutrient solution.—

Ca(NO₃)₂	...	0.096%
MgSO₄	...	0.016%
KNO₃	...	0.032%
KCl	...	0.024%
KH₂PO₄	...	0.008%

Preparation of Enzymes : (*a*) *Fumarase.—*Each gram of germinated seed was ground first in an ice cold mortar for 30 min. and then for 15 min. with double the volume of $M/20$-Na_2HPO_4 and finally extracted by pressing through muslin. The extract was used as fumarase. The whole procedure was conducted in cold.

In case of germinated seeds, the quantity of water absorbed during germination was determined, and calculated quantities of disodium hydrogen phosphate and water were added for extraction so that the final volume of extracting fluid was exactly double of the original weight of these seeds before germination. These precautions were necessary in order to compare the activity of fumarase in different enzyme solutions prepared under exactly identical conditions after different periods of germination.

(*b*) *Succino-dehydrogenase.—*Aliquot parts of the enzyme preparations used as fumarase (as described above) were dialysed in collodion sacks for 6 hours against distilled water in the cold and the dialysed solution was finally used as succino-dehydrogenase. The volume after dialysis was manipulated so as to enable the activity at different period of germination to be comparable. This dialysis was found essential in order to make the enzyme preparation free from donators.

FUMARASE

This enzyme converts fumaric acid into malic acid and *vice versa* by taking up or giving up one molecule of water. In presence of either fumaric or malic acid an equilibrium of these two substances in the ratio of fumaric acid / malic acid $= \frac{1}{3}$ is established by this enzyme. This enzyme has been found to be very widely distributed in animal tissues and plays a very fundamental rôle in the respiration of animal tissues. Consequently, investigations have been carried out with the object of studying the relative activity of this enzyme in the process of germination. The enzyme has been found to occur in pea, cucumber, barley and rice ; its activity increases with germination at least up to 7 days. The increase seems to be greater in the first few days than in the following days (see Fig. 1).

Sodium fumarate ($M/6$) ; Phosphate buffer ($M/40$, *p*H 7.0) ; Enzyme used coresponding to 10 g. of fresh ungerminated tissue (propyl alcohol and toluene were also added). Temperature 28°.

FIG. 1.

1. Ungerminated pea.   3. 5 days' germinated pea.
2. 3 days' germinated pea.   4. 7 days' germinated pea.
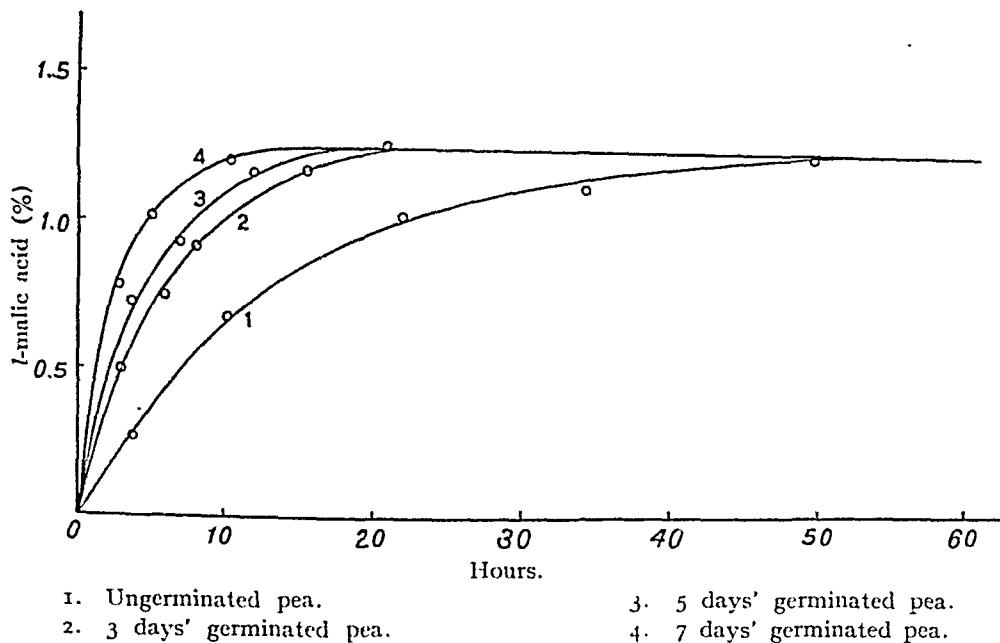
SUCCINO-DEHYDROGENASE

This enzyme dehydrogenating succinic acid and hydrogenating fumaric acid has long been known to be one of the most widely distributed enzymes in bacterial as also in animal and vegetable tissues. Much work has been done to show its rôle in the respiration of animal tissues and bacteria. But the significance of its occurrence in vegetable tissues is not understood.

In spite- of a large volume of work on this enzyme, much obscurity still exists as to the mechanism of its reactions. It is not definitely known if this enzyme requires the co-operation of any carrier or co-enzyme for its activity. But hydrogen carriers like di- and tri-phosphopyridine nucleotides do not take any part in this reaction. Another interesting observation is that this enzyme is specifically inhibited by malonic acid. Based on these facts theories on the rôle of succinic acid in respiration of animal tissues have been evolved which will be discussed later.

Recently Damodaran and Ramaswamy (13) have studied the activity of succino-dehydrogenase in germinating seeds. Using methylene blue as hydrogen acceptor they failed to get a considerable difference in the reduction time of the dyestuff between the control and the substrate in some cases. They however concluded that succino-dehydrogenase increases in the first few days of germination and then gradually decreases in activity. Using 2:6-dichlorophenolindophenol (higher redox potential) as hydrogen acceptor we have been able to get a remarkable difference in reduction periods of the dyestuff between the control and the substrate. It has been found that succino-dehydrogenase occurs even in the ungerminated seeds of

pea, cucumber, and barley. Its activity increases during the first three or four days of germination and then begins to decrease (Table I).

TABLE I.

Thunberg technique ; M/8 Phosphate buffer (pH 7.0) ; M/8 Sodium succinate ;
2 : 6-Dichlorophenolindophenol (1 in 6000). Total volume 2.5 cc. Temp. 37°.
Enzyme preparation used for each experiment corresponds to 0.14 g.
of fresh ungerminated seed.

Period of germina- tion (days)	Decolorisation time of dyestuff		Activity
	Experiment	Control	
0	31.5 min.	90 min.	2.84
3	7.25	60	8.28
5	14.75	90	6.11
7	13.5	59	4.37

RELATIVE ACTIVITY OF FUMARASE AND SUCCINO-DEHYDROGENASE

Fig. 1 and Table I show that both fumarase and succino-dehydrogenase simultaneously increase in the first few days of germination and then the latter begins to decrease while the former still continues to grow in activity.

In order to confirm these findings both succinic and fumaric acids were added separately in two reaction vessels to the same enzyme preparation, and the malic acid formed was estimated. We have already seen that the tissue contains succino-dehydrogenase which is capable of concerting succinic acid into fumaric acid. This fumaric acid again will be converted into malic acid by fumarase which is also present in the tissues. So by estimating malic acid formed in both these experiments we can determine the activity of both succino-dehydrogenase and fumarase in the same enzyme preparation. In fact, however, very little malic acid was formed even after a long period of incubation with succinic acid (Table II). The possibility of succino-dehydrogenase in plants requiring a carrier or acceptor of hydrogen which might be partially or entirely destroyed during the manipulation of plant tissues has not yet been excluded. This will be dealt with in a later communication.

TABLE II.

Germinated pea ground in cold mortar was used as enzyme preparation.
M/6 Phosphate buffer (pH 7.0), M/6 Sodium succinate and fumarate.

Period of germination	Reaction period	l-Malic acid formed from	
		Fumarate	Succinate
0 days	4 hours	0.67%	0.09%
3	4	0.66	0.06
6	4	0.80	0.05

INHIBITION OF PLANT TISSUE RESPIRATION

The inhibition of oxygen uptake by sliced vegetable tissues in presence of cyanide and malonate has also been studied. It has been found that both cyanide and malonate largely inhibit the respiration of vegetable tissue slices (Table III). The implication of these findings in the mechanism of vegetable tissue respiration will be discussed later.

TABLE III.

Tissue slices (1 g. fresh) were suspended in nutrient solution (pH 6.5) ; Both cyanide and malonate were adjusted to pH 6.5 ; Temperature 23°; Total volume 2.75 cc.

Time (mins.)	Oxygen uptake in c. mm.				
	Control without inhibitor	Cyanide		Malonate	
		M/20	M/10	M/20	M/10
30	118.7	74.99	30.14	106.4	64.5
60	271.1	113.8	54.25	234.2	175.3
90	402.4	164.9	81.37	346.3	272.2
120.	541.7	219.9	108.5	472.7	382.9

DISCUSSION

We have already described the rôles of C_4-dicarboxylic acids and consequently that of fumarase and succino-dehydrogenase in the respiration of animal tissues. Their occurrence in vegetable tissues naturally makes one think of their significance in general metabolism of the plant.

From the occurrence of fumarase and succino-dehydrogenase in vegetable tissues and their increased activity during germination (which suggest their participation in the life-process of the plant), we are of opinion that they play the same part in the respiration of vegetable tissues as in that of animal tissues (Chibnall, 14). The experimental evidences reported here seem to indicate that only a fraction (about 30%) of the total vegetable tissue respiration is catalysed by C_4-dicarboxylic acids. This theory is supported by the large inhibition of respiration of those sliced tissues by cyanide and malonate. In none of the cases however total inhibition of respiration was noted. The classical researches of Warburg and of Szent-Györgyi have clearly demonstrated the mechanism of inhibition of respiration by cyanide and malonate. Cyanide poisons respiration by inhibiting the reoxidation of cytochrome by molecular oxygen activated by "Atmungsferment" or cytochrome oxidase whereas malonate poisons respiration by inhibiting specifically succino-dehydrogenase. Both these enzymes namely cytochrome oxidase (Bhagat and Hill, 10) and succino-dehydrogenase have been found to occur in vegetable tissues. Inhibition of vegetable tissue respiration by cyanide and malonate can, therefore, be attributed to the poisoning of these two enzymes, thereby supporting the theory that a part of vegetable tissue respiration is dependent on these two enzymes.

The fact that cyanide inhibition is much greater than malonate inhibition suggests that cytochrome is not only reduced by succino-dehydrogenase (which is inhibited by malonate) but also by some other malonate-insensitive enzymes like "diaphorase" (Adler *et al*, 8). It is still a controversial point whether cytochrome is replaced by any other carrier in higher plants, in spite of occurrence of cytochrome oxidase which might oxidase other carriers as well. A portion (about 20%) of vegetable tissue respiration is not inhibited by cyanide when sliced tissues are used as plant material. Investigations are in progress to study the effect of slicing on the normal respiration of plants and to devise some method for studying the micro-respiration of isolated vegetable tissues.

During the first few days of germination enhanced activity of both succino-dehydrogenase and fumarase is necessary in order to carry on the intensive respiration and also to help the formation of asparagine which has been found to increase during germination of seeds (Chibnall, 14). This amide is formed either from oxalacetic acid (Reaction I) formed by dehydrogenation of malic acid or from fumaric acid (Reaction II), the first mechanism being more in accord with the recent views of the synthesis of amino-acids (Adler *et al*, 15, 16 ; Euler *et al*, 17 ; Chibnall, 14).

REACTION I.

$$
\begin{array}{ccccccc}
\text{COOH} & & \text{COOH} & & \text{COOH} & & \text{COOH} \\
| & & | & & | & -H_2 & | \\
\text{CO} & +NH_3 \;\rightleftarrows & \text{C}=\text{NH} & & \text{C}=\text{NH} & \rightleftarrows & \text{CHNH}_2 \\
| & & | & +H_2O & | & +H_2 & | \\
\text{CH}_2 & & \text{CH}_2 & & \text{CH}_2 & & \text{CH}_2 \\
| & & | & & | & & | \\
\text{COOH} & & \text{COOH} & & \text{COOH} & & \text{COOH} \\
& (1) & & & & (2)
\end{array}
$$

$$
\begin{array}{ccc}
\text{COOH} & & \text{CONH}_2 \\
| & & | \\
\text{CHNH}_2 & +NH_3 \;\rightleftarrows & \text{CHNH}_2 \quad +H_2O \\
| & & | \\
\text{CH}_2 & & \text{CH}_2 \\
| & & | \\
\text{COOH} & & \text{COOH} \\
& (3)
\end{array}
$$

REACTION II.

$$
\begin{array}{cccccc}
\text{COOH} & & \text{COOH} & \text{COOH} & & \text{CONH}_2 \\
| & +NH_3 & | & | & +NH_3 \;\rightleftarrows & | \\
\text{CH} & \rightleftarrows & \text{CHNH}_2 & \text{CHNH}_2 & & \text{CHNH}_2 \\
\| & -NH_3 & | & | & & | \quad +H_2O \\
\text{CH} & & \text{CH}_2 & \text{CH}_2 & & \text{CH}_2 \\
| & & | & | & & | \\
\text{COOH} & & \text{COOH} & \text{COOH} & & \text{COOH} \\
& (1) & & & (2)
\end{array}
$$
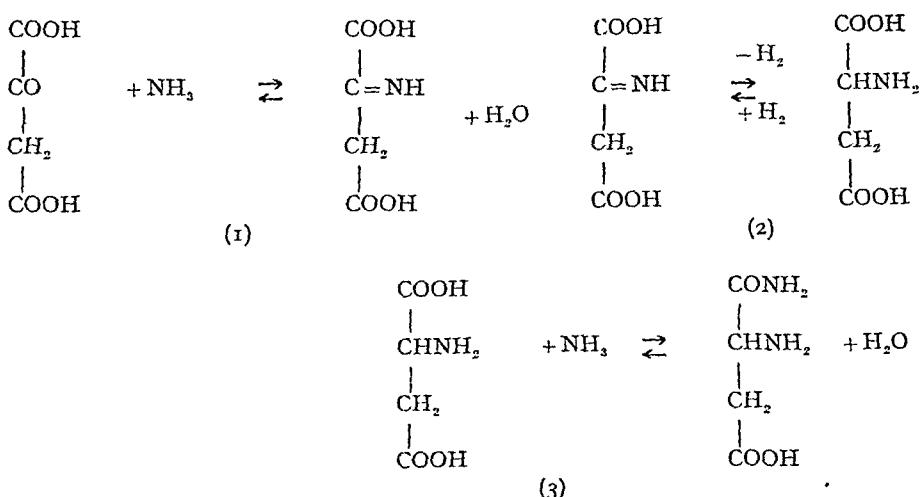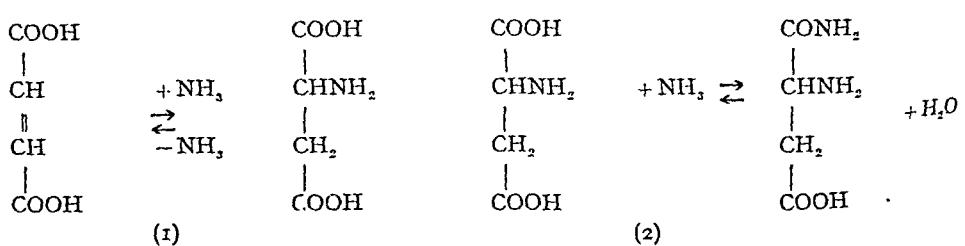
In Reaction I the equilibrium is in favour of aspartic acid. By this reaction plants bind up free ammonia which is formed by other reactions. Asparagine is thus a store-house of ammonia which otherwise might have poisoned the living system. This ammonia is released from asparagine and consequently from aspartic acid according to necessity.

The activity of fumarase is unimpaired and continues to grow slowly even after the activity of succino-dehydrogenase has diminished, as it is necessary to convert at once all the fumaric acid formed from sources other than succinic acid, like aspartic acid, into malic acid which is dehydrogenated to oxalacetic acid.

Aspartic acid

Succinic acid $\rightleftarrows$ Fumaric acid $\rightleftarrows$ Malic acid $\rightleftarrows$ Oxalacetic acid
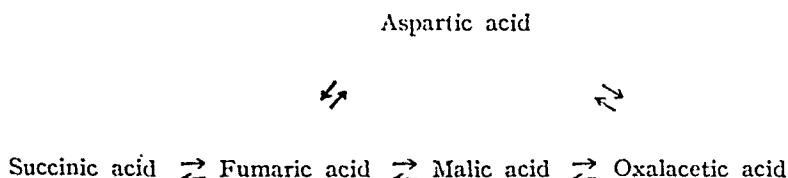
Succino-dehydrogenase is capable of carrying respiration even if present in small amount when all the metabolic cycles are once in action and the diminished activity of succino-dehydrogenase after a few days of germination might be due to the fall of total respiration after the first few days of germination when it is maximum (Hafenrichter, 18).

The oxalacetic acid formed from malic acid by malic dehydrogenase which also occurs in vegetable tissues (Euler and Weichert, 19) is also utilised in many other reactions excepting that of carrying hydrogen from the metabolite or "foodstuff" in respiration of vegetable tissues as presented in this paper. The following scheme shows the various functions of oxalacetic acid in vegetable tissues.
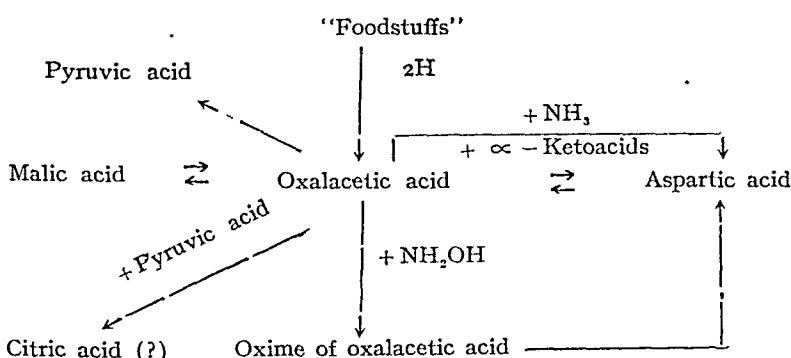
From the above two schemes it is clear that either oxalacetic acid or fumaric acid, preferably the former, must be formed in order that asparagine may be synthesised. The rôle of citric acid which is also largely found in vegetable tissues has not been discussed here as conflicting results have

2

been reported by various authors and our present investigations have not been directed to that aim. In this paper we have also omitted the mechanism of glutamine formation which will be dealt with later.

We are grateful to Dr. D. M. Bose for his interest in this work.

SUMMARY

1. Fumarase has been found to occur both in germinated and in ungerminated seeds of pea, cucumber, barley and rice, being the least in rice. The enzyme increases in activity with germination at least up to 7 days

2. Succino-dehydrogenase is present in pea, cucumber and barley, either germinated or ungerminted. The enzyme increases in the first three or four days of germination and then diminishes gradually.

3. Very little or no *l*-malic acid is formed when succinic acid is added to minced vegetable tissues.

4. Oxygen uptake by slices of germinated pea was largely inhibited both by cyanide and malonate, the inhibition being much greater in the former case.

5. Theories of C_4-dicarboxylic acids and cytochrome taking part in vegetable tissue respiration and the mechanism of formation of asparagine have been discussed.

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A STUDY OF THE VITAMIN-A NUTRITION OF SOME HUMAN SUBJECTS WITH THE BIOPHOTOMETER

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(Received for publication, April 4, 1941)

It is an established fact that visual purple of the rods is a derivative of vitamin A. When light falls on the retina visual purple is bleached and its rate of regeneration, for which scotopic vision is responsible, depends on the vitamin A concentration of the blood and hence on the vitamin A nutrition of the body. With a defective intake of vitamin A the ability to see a dimly illuminated object in a dark room is diminished or the rate of recovery of such power, after a period of exposure to bright light, is delayed. This forms the basis of the dark adaptation test by which the vitamin A status of the body is assessed.

Harris and Abbasy (1), after making a critical examination of most of the important work on dark adaptation, put forward the view that 'there is a correlation between diminished dark-adaptation and deficiency of vitamin A'. Jeans *et al* (2) on the basis of this correlation have made an instrument called Biophotometer with which they have assessed the vitamin-A nutrition of human subjects. Several observers (3-7), however, have questioned the reliability of the biophotometer as an efficient instrument for performing dark adaptation test, while others (8, 9) consider that provided the instrument is calibrated frequently it is adequate for measuring marked disadaptation in adults. In the biophotometer there is a central metal screen in which is punched out five spots in a quincunx. The light intensity on the screen is varied by altering the resistance in series of a filament lamp, which causes an alteration in its intensity. The instrument makers furnish a set of calibrations in terms of millifoot-candles of illumination in front of the illuminated central dot of the quincunx. These calibrations are made with a photronic cell fitted with a visual correction colour filter. The current output corresponding to the incident illumination is converted into equivalent millifoot-candles of illumination by means of data supplied with the photronic cell. As the intensity of light illuminating the central screen may vary in course of time it is essential that the instrument should be calibrated frequently. In the present investigation the instrument was calibrated both before the beginning and after the end of the experiments and it was observed that the results agreed with the data supplied by the makers.

EXPERIMENTAL

The instrument was installed in a dark room. The observations were made by determining the amount of light necessary for the subject to see three of the five dots of a disc of five spots or quincunx, when the light transmitted through the holes was of decreasing intensity from the left to the right of the quincunx.

A series of light threshold measurements were made on each subject in accordance with the procedure previously described by Jeans *et al* (2). The test period consisted of successive measurements of the threshold of light sensitivity at five minutes interval during a ten-minute period of dark adaptation, immediately after three-minute period of adaptation to the bright light of the large diffusing screen and at three-minutes interval during a second dark adaptation period of nine minutes. The measurements are expressed in terms of millifoot-candles of illumination at the eye position, the eye of the subject being directly in front of and at a distance of 30 cm. from the illuminated dots of the screen. No value was accepted unless the subject recorded the same or a neighbouring reading on repeated examinations. Subjects included male persons engaged in research, post-graduate students, assistants in the workshop, servants, lecturers and students from a local school. In the following tables data presented are confined to the measurements taken within an interval of approximately 20 seconds following the adaptation to the standard bright light and the final measurement was taken during the recovery period (Table I).

TABLE I.

Serial no. of subject.	Age in years.	Threshold values expressed in millifoot candles.	
		20 Sec. after 3 min. exposure to bright light.	At the end of 9 mins. recovery period.
Research Workers.			
1.	23	0.222	0.0633
2.	30	0.110	0.0294
3.	28	0.0633	0.0059
4.	34	0.119	0.0146
5.	25	0.273	0.0180
6.	26	0.238	0.0110
7.	25	0.388	0.1370
8.	27	0.478	0.0233
9.	26	0.137	0.0096
10.	23	0.388	0.0068
11.	24	0.137	0.0445
12.	32	0.0894	0.0059
13.	23	0.206	0.0063
14.	26	0.222	0.0083
15.	24	0.273	0.0388
16.	30	0.388	0.0127
17.	24	0.168	0.0078

TABLE I.—(contd.)

Serial no. of subject.	Age in years.	Threshold values expressed in millifoot candles.	
		20 Sec. after 3 min. exposure to bright light.	At the end of 9 mins. recovery period.
18.	23	0.314	0.0180
19.	25	0.362	0.0083
20.	25	0.238	0.0059
21.	29	0.256	0.0096
22.	28	0.206	0.0059
23.	28	0.206	0.0063
24.	23	0.778	0.0193
25.	25	0.0778	0.0059
26.	24	0.119	0.0063
27.	27	0.137	0.0059
Post-graduate Students.			
28.	22	0.256	0.0068
29.	22	0.337	0.0078
30.	23	0.388	0.0096
31.	22	0.103	0.0059
32.	21	0.362	0.0063
33.	21	0.677	0.0778
34.	21	0.416	0.0273
35.	21	0.137	0.0059
36.	21	0.0677	0.0137
37.	20	0.110	0.0059
38.	21	0.206	0.0096
39.	21	0.238	0.0059
40.	22	0.337	0.0157
41.	21	0.0959	0.0059
42.	22	0.137	0.0157
43.	21	0.273	0.0089
44.	22	0.222	0.0083
45.	22	0.222	0.0110
46.	22	0.127	0.0059
47.	23	0.0314	0.0059
48.	23	0.445	0.0157
49.	25	0.256	0.0119
50.	21	0.362	0.0083
51.	23	0.127	0.0059
52.	23	0.445	0.0089
53.	21	0.206	0.0096
54.	23	0.256	0.0089
55.	19	0.206	0.0089
56.	21	0.168	0.0103
57.	21	0.238	0.0089
58.	20	0.256	0.0089
59.	22	0.273	0.0089
60.	22	0.137	0.0072
61.	20	0.206	0.0127
62.	19	0.206	0.0089

TABLE I.—(contd.)

Serial no. of subject.	Age in years.	Threshold values expressed in millifoot candles.	
		20 Sec. after 3 min. exposure to bright light.	At the end of 9 mins. recovery period.
63.	20	0.168	0.0078
64.	22	0.168	0.0078
65.	23	0.0677	0.0059
66.	34	0.206	0.0089
67.	19	0.273	0.0119
68.	19	0.157	0.0083
69.	21	0.168	0.0119
70.	20	0.337	0.0059
71.	29	0.273	0.0059
72.	23	0.445	0.0168
73.	23	0.222	0.0063
74.	21	0.510	0.0119
75.	19	0.193	0.0168
76.	21	0.193	0.0119
77.	21	0.157	0.0063
78.	23	0.388	0.0089
79.	24	0.294	0.0256
80.	22	0.193	0.0078
81.	22	0.137	0.0059
82.	28	0.362	0.0238
Workshop Assistants and Bearers.			
83.	32	0.222	0.0059
84.	26	0.256	0.0059
85.	20	0.273	0.0063
86.	41	0.168	0.0238
87.	20	0.193	0.0059
88.	31	0.337	0.0059
89.	25	0.193	0.0059
90.	44	0.294	0.0157
91.	30	0.206	0.0103
92.	30	0.294	0.0110
93.	25	0.180	0.0362
94.	18	0.550	0.0590
95.	24	0.0959	0.0238
96.	28	0.168	0.0146
97.	22	0.222	0.0127
98.	25	0.314	0.0256
99.	20	0.238	0.0238
100.	23	0.314	0.0168
101.	34	0.256	0.0180
102.	22	0.193	0.0238
103.	22	0.0388	0.0168
104.	27	0.314	0.0137
105.	18	0.0238	0.0059
106.	28	0.362	0.0146
107.	40	0.337	0.0137

TABLE I.—(contd.)

Serial no. of subject.	Age in years.	Threshold values expressed in millifoot candles.	
		20 Sec. after 3 min. exposure to bright light.	At the end of 9 mins. recovery period.
108.	30	0.256	0.0119
109.	26	0.416	0.0222
110.	30	0.238	0.0103
111.	35	0.337	0.0137
112.	55	0.590	0.0157
113.	22	0.337	0.0068
114.	19	0.137	0.0059
115.	22	0.0388	0.0089
116.	24	0.157	0.0059
117.	18	0.294	0.0096
118.	24	0.273	0.0059
119.	22	0.294	0.0238
120.	21	0.193	0.0059
121.	42	0.193	0.0083
122.	38	0.590	0.0083
123.	45	0.388	0.0238
124.	19	0.193	0.0110
Lecturers.			
125.	40	0.238	0.0416
126.	41	1.10	0.0314
127.	33	0.222	0.0063
School Students.			
128.	8	0.273	0.0137
129.	14	0.222	0.0127
130.	15	0.294	0.0089
131.	15	0.0834	0.0059
132.	15	0.238	0.0119
133.	17	0.180	0.0180
134.	17	0.193	0.0127
135.	14	0.222	0.0238
136.	9	0.677	0.0222
137.	13	0.834	0.2940
138.	14	0.834	0.0834

The effect of administration of cod liver oil to 6 frankly deficient cases and 16 supposedly normal cases according to the standard of Booher *et al* (8) was studied. The above individuals were given for two months a daily dose of cod liver oil containing 5000—10000 international units of vitamin A as determined by a Spekker-spectrophotometer (Adam-Hilger) together with 10 cc. of lemon juice, the vitamin C in which is said to help in the assimilation of vitamin A (10). The results are given in Table II. The estimation of vitamin A in the cod-liver oil was made on the unsaponifiable fraction of the 'oil, prepared according to the recommendations of the Medical

Research Council (11). The values found spectrophotometrically (Fig. 1) were converted into international units of vitamin A from the following formula.

$$\text{(at 328 m}\,\mu\text{) E } \frac{1\%}{1 \text{ cm.}} \times 2200 = 1430 \text{ international units per g.}$$

FIG. 1.

Wave-length in mμ
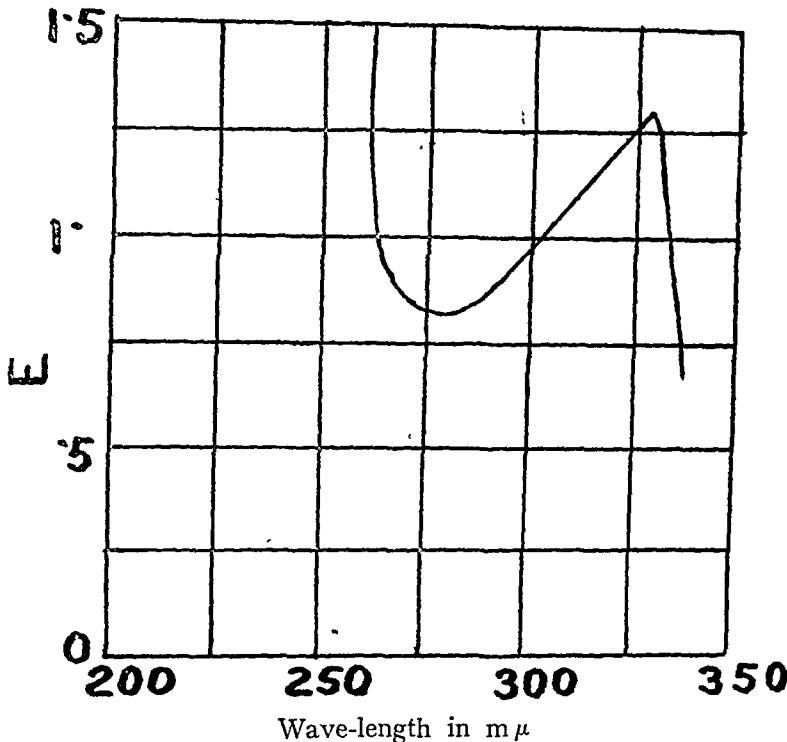
TABLE II.

Threshold values expressed in millifoot candles of twenty-two subjects before and after daily administration of cod liver oil with lemon juice for two months.

Subject.	Age in years.	Wt. in pounds.	Threshold values before administration of cod liver oil.		Threshold values after administration of cod liver oil.	
			(1)	(2)	(1)	(2)
S.R.	9	56	0.677	0.0222	0.127	0.0127
B.M.	13	70	0.834	0.294	0.337	0.0362
N.S.	14	82	0.834	0.0834	0.273	0.0180
S.S.	23	136	0.778	0.0193	0.238	0.0072
A.G.	21	142	0.725	0.0388	0.337	0.0180
S.N.	21	134	0.778	0.0550	0.137	0.0096

TABLE II.—(contd.)

Subject	Age in years	Wt. in pounds.	Threshold values before administration of cod liver oil.		Threshold values after administration of cod liver oil.	
			(1)	(2)	(1)	(2)
R.D.	21	140	0.362	0.0083	0.193	0.0083
G.S.	22	106	0.388	0.0119	0.222	0.0059
B.P.	21	134	0.388	0.0068	0.193	0.0059
S.B.	21	126	0.478	0.0273	0.119	0.0119
S.G.	20	106	0.416	0.0362	0.168	0.0388
V.G.	19	116	0.416	0.0063	0.157	0.0078
G.B.	19	104	0.416	0.0157	0.137	0.0168
P.G.	21	102	0.416	0.0168	0.168	0.0076
B.B.	23	100	0.445	0.0059	0.238	0.0089
N.M.	20	164	0.445	0.0677	0.273	0.0337
A.R.	19	118	0.314	0.0078	0.193	0.0068
N.D.	22	130	0.388	0.0089	0.180	0.0072
S.C.	21	140	0.388	0.0209	0.256	0.0110
S.R.	22	110	0.388	0.0089	0.157	0.0059
B.S.	25	115	0.388	0.0127	0.337	0.0209
B.D.	23	115	0.445	0.0157	0.337	0.0103

(1) First reading taken 20 sec. after exposure to bright light.
(2) Last reading taken 9 min. after exposure to bright light.

DISCUSSION

According to Booher et al (8) the border-line regions for the threshold value 20 sec. after 3 minutes' exposure to bright light and for the threshold at the end of the 9 minute recovery period, separating the normal from the vitamin A deficient subjects, are around 0.70 and 0.06 millifoot-candles respectively. According to their standards individuals No. 1, 24, 33, 94, 125, 136, 137, 138 out of a total of 138 examined (Table I) are deficient in vitamin A. Except the individual No. 136 who was night blind and cured of the disease after taking cod-liver oil none of the vitamin A deficient individuals showed any other symptoms of vitamin A deficiency. From Table II, however, it is apparent that after the administration of cod-liver oil the threshold values for both the deficient (first six) and supposedly normal cases are diminished, showing improvement in the dark adaptation. It would thus appear that those individuals, who according to Booher's standards might be considered to be normal, were not in optimum condition with regard to the vitamin A nutrition of the body. It is also observed that even after administration of 4 to 8 g. of cod liver oil containing 5000 to 10000 units of vitamin A per day, the threshold value still goes down indicating that the optimum requirements of vitamin A have not been supplied. The present investigation shows that major and frank symptoms of vitamin A deficiency are not very common in the adult population of Calcutta. Three of the

eleven boys ranging from 8 to 15 years, who were examined in a local School in Calcutta showed, however, deficient dark adaptation according to Booher's standard. The work is in progress in order to find the optimum requirement of vitamin A of Indians of different age groups.

SUMMARY

(1) One hundred and thirty-eight individuals of different strata of society have been examined with the biophotometer to assess their level of vitamin A nutrition. Of 127 individuals of age varying between 19 and 55 only five showed deficient dark adaptation according to the standards of American workers. Of the 11 school students (age varying between 8 and 15 years) 3 showed deficient dark adaptation. One of the students was suffering from night blindness and was cured of it after being supplemented with cod liver oil.

(2) Clinical signs of vitamin A deficiency were absent in all the subjects examined.

(3) Supplements of cod liver oil with lemon juice greatly diminished the threshold values showing an improved dark adaptation in deficient, as well as in normal cases. This suggests that the subjects studied were not in optimum condition with respect to vitamin A.

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DISTRIBUTION OF FREE AND BOUND ASCORBIC ACID AND HÆMOGLOBIN IN BLOOD OF NORMAL VERTEBRATES

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It has been shown previously from this laboratory (1) that many plant foodstuffs contain free as well as bound ascorbic acid (ascorbigen). Similar investigations have been carried out by the same authors (2) concerning the distribution of bound ascorbic acid in different animal tissues also. The free and bound ascorbic acid contents of the liver and muscle tissues of some Bengal fresh-water fish have also been studied by one of us (3).

The ascorbic acid level of the blood has been taken as an index of the "nutritional state" with respect to vitamin C by some workers. It has been found that in clinical scurvy the ascorbic acid content of blood reaches low levels (0.1 – 0.3 mg. per 100 cc.) which may be restored to normal by the oral or intravenous administration of ascorbic acid. It was of interest to know the concentration of both free and combined ascorbic acid in blood under normal condition, and as there is some evidence indicating that ascorbic acid may have some relation to blood formation, it was considered desirable to study also the hæmoglobin content of the blood at the same time.

EXPERIMENTAL

Drawing of blood :

Ten cc. of blood were drawn out from the cubital vein of young men varying in age from 21 to 33 years. In the case of rabbits, 10 cc. of blood were drawn out from the heart of each animal. In the case of guineapigs and rats, however, as sufficient blood could not be conveniently obtained from one animal, 2.5 cc. blood were drawn from the heart of each animal and the blood from 4 animals was pooled. Blood of the pigeon was obtained from the axillary vein in sufficient quantities. In the case of fowls, blood was obtained from subclavian vein.

Estimation of hæmoglobin :

The hæmoglobin content of the blood was determined by Shalli's hæmatometer.

Estimation of free and combined ascorbic acid :

The estimations were carried out as follows according to the general method described previously (4). Out of 10 cc. of the collected blood, 4 cc. were taken in a centrifuge tube (50 cc.) and 2 cc. of water were added to hæmolyse it. The hæmolysed blood was treated with 1 cc. of glacial acetic acid and then with 3 cc. of 20% trichloroacetic acid to deproteinise the blood. The mixture was then well shaken and centrifuged. The clear centrifugate along with the washings of the precipitate was taken in a 50 cc. conical flask, sulphuretted hydrogen was passed for 15 minutes in the cold and it was then removed by passing carbon dioxide. The volume was then made up to 15 cc. and the titration was made as usual by means of 2:6-dichlorophenol-indophenol.

Another 4 cc. of the blood from the same sample was treated as mentioned above but sulphuretted hydrogen was passed into the protein-free filtrate for 15 minutes under hot condition. The results are given in Tables I, II and III.

In these estimations, aliquots were treated in the usual manner with ascorbic acid oxidase in order to obtain true ascorbic acid values.

As in separate experiments it was found that there was no difference in the results obtained by the direct indophenol titration of the protein-free filtrate and the titration made after the treatment with cold sulphuretted hydrogen, it was concluded that normal blood does not contain any dehydro-ascorbic acid.

TABLE I

Ascorbic acid, ascorbigen and hæmoglobin content of normal human subjects.

Age	Body weights.	Hb.	Free ascorbic acid (per 100 cc. blood)	Ascorbigen. (per 100 cc. blood)	Total (ascorbic acid + ascorbigen.)
21	54 kg.	90%	1.62 mg.	0.54 mg.	2.16 mg.
22	61	85	1.33	0.52	1.85
23	58	98	1.08	0.77	1.85
22	63	99	0.92	0.61	1.53
21	55	91	0.81	0.81	1.62
20	57	95	1.00	1.16	2.16
23	69	87	0.81	0.64	1.44
33	74	100	0.86	0.62	1.48
26	70	80	0.60	0.60	1.20

TABLE II

Ascorbic acid, ascorbigen and hæmoglobin content
of normal guinea-pigs, rats and rabbits.

Source of blood.	Body weights.	Hb.	Free ascorbic acid (per 100 cc. blood)	Ascorbigen (per 100 cc. blood)	Total (ascorbic acid + ascorbigen).
Guinea-pigs	279—246 g.	75%	1.04 mg.	1.56 mg.	2.60 mg.
	255—244	90	0.74	1.11	1.85
	257—238	90	0.80	1.28	2.08
Rats.	72— 63	102	0.56	1.19	1.75
	81— 72	96	0.76	0.70	1.46
Rabbits.	1290	84	0.74	0.56	1.30
	1345	78	0.47	0.19	0.66
	1370	70	0 45	0.10	0.55
	1240	90	0.76	0.54	1.30
	1417	94	0.61	0.65	1.26
	1380	97	0.74	0.59	1.33

TABLE III

Ascorbic acid, ascorbigen and hæmoglobin content
of normal pigeons and fowls.

Source of blood.	Body weights.	Hb	Free ascorbic acid (per 100 cc. blood)	Ascorbigen (per 100 cc. blood)	Total (ascorbic acid + ascorbigen)
Pigeon	300 g.	86%	0.92 mg.	0.80 mg.	1.72 mg.
	313	91	0.64	0.72	1.35
	328	82	1.08	0.61	1.69
	340	88	0.68	0.84	1.52
	321	81	0.31	1.60	1.91
	350	87	0.63	0.79	1.42
Fowl	917	83	0.73	1.51	2.24
	900	85	0.88	1.19	2.07
	987	96	0.31	1.20	1.51
	1010	91	0.58	1.34	1.92
	961	80	0.35	1.24	1.59
	952	83	0.33	1.62	1.95

SUMMARY

Free ascorbic acid, ascorbigen and hæmoglobin levels of the blood of different species.

Source of blood.	Variation in hæmoglobin.	Mean Hb.	Variation of free ascorbic acid (mg. per 100 cc.)	Mean free ascorbic acid (mg. per 100 cc.)	Variation of ascorbigen (mg. per 100 cc.)	Mean ascorbigen expressed as ascorbic acid (mg. per 100 cc.)	Mean ascorbic acid + ascorbigen (mg. per 100 cc.)
Human adults	70—100	90.5	0.60—1.62	1.00	0.52—1.16	0.69	1.69
Guinea-pigs	75— 90	85.2	0.75—1.40	0.86	1.11—1.56	1.31	2.17
Rabbits	72— 97	85.5	0.45—0.76	0.58	0.10—0.65	0.44	1.02
Rats	96—102	99.0	0.56—0.76	0.66	0.70—1.19	0.94	1.60
Fowls	80— 91	87.0	0.33—0.88	0.56	1.19—1.62	1.19	1.75
Pigeons	81— 91	86.0	0.31—1.08	0.71	0.61—1.60	0.89	1.60

From the above results it is clear that ascorbigen (combined ascorbic acid) is a normal constituent of the blood of the human, the rabbit, the guineapig, the rat, the fowl and the pigeon. There are fairly large variations in ascorbic acid and ascorbigen content among the individual members of the same species and it is not clear whether the variations between species and species have any significance.

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RELATION BETWEEN BLOOD ASCORBIC ACID, ASCORBIGEN AND HÆMOGLOBIN AT DIFFERENT STAGES OF SCURVY IN EXPERIMENTAL GUINEAPIGS

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That some plant as well as animal tissues contain a part of ascorbic acid in the combined state (ascorbigen) has been shown in previous papers (1—4). As in one of these papers (4) we have shown that blood contains both ascorbic acid and ascorbigen, it was of importance to know how their concentrations in blood would be affected by scurvy. Further it has long been recognised (5) that scurvy is accompanied by a fall in the hæmoglobin level of blood. It was therefore considered desirable to carry out these estimations also along with the studies of the ascorbic acid and ascorbigen content of blood. Such investigations are likely to throw some light on the rôle of vitamin C in blood formation, if any, and also on the biochemical relationship of ascorbigen to ascorbic acid. In the present paper these studies, concerning guineapigs, in the normal, pre-scorbutic and scorbutic stages are reported.

EXPERIMENTAL

Seventy-two healthy male guineapigs, all having approximately the same weight, were fed on the same normal diet of green grass and germinating gram for 7 days prior to the actual experiment. They were then divided into 6 groups having twelve in each group. The blood hæmoglobin, ascorbic acid and ascorbigen were determined by the technique previously described (4).

One group was taken to provide the normal figures for blood ascorbic acid, ascorbigen and hæmoglobin after 7 days' feeding on the normal diet and then this group was rejected. The other five groups were kept on the scorbutic diet after 7 days' normal feeding.

The determinations of the blood ascorbic acid, ascorbigen and hæmoglobin with three of these five groups were carried out respectively after 7, 15 and 21 days from the commencement of the scorbutic ration. The two remaining groups were fed after the 21st day with a supplement of 1.0 mg. of ascorbic acid per animal per day. The blood was pooled from the hearts of four animals in each group for these determinations as has been previously described (4). The results are given in Table I and the mean values are summarised in Table II.

TABLE I.

No. of animals.	Range of initial body wts. (g.)	Nature of diet.	Hæmoglobin.	Free ascorbic acid per 100 cc. of blood.	Ascorbigen expressed as ascorbic acid per 100 cc. of blood.	Free and combined ascorbic acid per 100 cc. of blood.
4	246—252	Normal diet for 7 days	75%	1.04 mg.	1.56 mg.	2.60 mg.
4	244—255	,,	90	0.74·	1.11	1.85
4	238—257	,,	90	0.80	1.28	2.08
4	233—245	Scorbutic diet for 7 days	87	0.81	1.27	2.08
4	246—253	,,	88	0.76	1.08	1.84
4	141—247	,,	75	0.74	0.56	1.30
4	236—245	Scorbutic diet for 14 days	73	0.65	0.65	1.30
4	231—244	,,	68	0.59	0.71	1.30
4	242—251	,,	65	0.66	0.74	1.40
4	236—253	Scorbutic diet for 21 days	58	0.42	0.28	0.70
4	242—248	,,	55	0.43	0.22	0.65
4	238—252	,,	61	0.41	0.24	0.65
4	236—251	Scorbutic diet for 28 days with the supplement of 1 mg. of vitamin C per day from the 22nd day.	70	0.62	0.63	1.22
4	246—253	,, ,,	68	0.64	0.75	1.39
4	228—243	,, ,,	66	0.53	0.72	1.25
4	240—255	Scorbutic diet for 36 days with the supplement of 1 mg. of vitamin C per day from the 22nd day.	76	0.81	1,00	1.81
4	243—250	,, ,,	78	0.92	0.93	1.85
4	236—251	,, · ·	74	0.82	1.13	1.95

As Tables I and II indicate, with continued feeding on a scorbutic diet there is a fall in the concentration of blood hæmoglobin. It was of interest to know whether vitamin C deficiency affects this hæmoglobin level only indirectly, as vitamin C deficiency tends to diminish the food-intake and therefore the iron-intake. In order to settle this question, two groups of guineapigs having twelve animals in each group were used. One group was put on a scorbutic diet and the other group on a scorbutic diet supplemented by 2 mg. ascorbic acid per animal per day. The food-intake of the animals were measured throughout and it was observed that the

food-intake suffered an abrupt fall about the 21st day on the scorbutic diet. The animals of the group receiving the scorbutic diet supplemented by ascorbic acid were given exactly the same amounts of diet which were consumed by the corresponding animals on the scorbutic diet during the past 24 hours. In this way the basal diets of the animals receiving ascorbic acid were restricted to the amounts voluntarily taken by the scorbutic animals. The hæmoglobin levels of blood obtained by heart puncture of all these individual animals were measured at the beginning and at the end of the experimental period of 24 days, by which time the animals were very weak and nearly dying. The results, as given in Tables III and IV, show that animals receiving vitamin C but restricted amounts of diet, nevertheless show normal blood hæmoglobin levels, while the scorbutic animals, eating the same quantities of diet exhibit a considerably lowered blood hæmoglobin concentrations.

TABLE II.

Mean values of blood hæmoglobin, ascorbic acid and ascorbigen of guineapigs under normal, pre-scorbutic and scorbutic conditions and after oral administration of vitamin C from the 22nd day of a scorbutic ration.

No. of animals.	Nature of diet.	Hæmoglobin.	Free ascorbic acid per 100 cc. of blood.	Ascorbigen expressed as ascorbic acid per 100 cc. of blood.	Free and combined ascorbic acid per 100 cc. of blood.
12	Normal diet for 7 days.	85%	0.86 mg.	1.31 mg.	2.17 mg.
12	Scorbutic diet for 7 days.	83	0.77	0.94	1.71
12	Scorbutic diet for 14 days.	69	0.63	0.70	1.33
12	Scorbutic diet for 21 days.	58	0.42	0.25	0.67
12	Scorbutic diet for 28 days with supplement of 1.0 mg. of ascorbic acid per day from the 22nd day.	68	0.59	0.69	1.28
12	Scorbutic diet for 36 days with supplement of 1.0 mg. of ascorbic acid per day from the 22nd day.	76	0.85	1.02	1.87

4

TABLE III.

Amounts of diets consumed by twelve scorbutic animals which were supplied to the corresponding animals receiving vitamin C for a period of 24 days.

Amounts of diet in g. consumed by animals no. 1-12.

Date.	1	2	3	4	5	6	7	8	9	10	11	12
7.3.41	22.0	24.0	17.3	14.0	11.6	12.8	16.5	20.3	23.0	12.8	22.2	19.6
8.3.41	19.3	22.5	15.8	21.5	16.2	16.0	14.6	18.6	19.2	15.3	17.8	17.3
9.3.41	17.2	22.2	15.3	15.2	15.0	21.2	14.7	18.8	18.0	14.2	21.0	12.6
10.3.41	18.0	23.2	19.3	20.1	15.0	16.0	16.0	19.0	22.3	16.9	16.3	18.0
11.3.41	18.0	18.1	18.6	18.3	17.3	12.0	15.5	18.0	20.3	16.8	18.0	16.0
12.3.41	19.5	19.3	18.5	18.8	14.9	17.3	15.0	17.0	21.0	15.8	19.1	15.1
13.3.41	18.0	18.6	14.0	17.5	15.2	17.6	15.1	17.9	23.0	15.6	19.3	16.3
14.3.41	20.2	20.5	15.0	21.0	15.3	15.5	16.3	19.2	22.2	14.2	17.7	16.6
15.3.41	19.3	19.0	10.1	19.0	19.6	17.7	14.7	21.1	18.1	17.6	16.0	15.2
16.3.41	20.1	24.2	22.0	14.3	14.5	17.7	17.0	17.3	19.6	16.2	16.6	14.0
17.3.41	19.1	26.0	19.3	13.0	13.7	17.2	14.7	15.0	19.2	16.0	16.2	19.0
18.3.41	21.2	20.0	20.1	15.0	15.0	15.1	15.6	16.2	19.6	16.0	19.0	19.3
19.3.41	20.0	20.2	15.3	15.6	15.0	10.0	15.2	18.0	21.2	15.2	17.5	16.5
20.3.41	20.2	20.1	16.6	14.5	16.2	10.2	18.1	18.6	20.0	14.1	15.3	18.7
21.3.41	20.1	22.2	16.6	16.3	14.0	15.0	14.0	19.5	20.3	17.3	18.8	19.2
22.3.41	19.8	20.6	16.5	15.2	15.1	14.0	14.5	18.2	17.4	13.3	19.5	15.0
23.3.41	17.6	17.8	14.7	19.9	14.2	10.3	14.2	17.7	18.4	14.5	17.3	14.4
24.3.41	15.0	16.5	15.8	16.3	12.0	10.6	13.3	16.2	17.0	15.8	16.7	13.2
25.3.41	15.5	18.5	11.0	15.2	15.9	10.5	15.8	16.1	18.0	12.6	15.1	16.0
26.3.41	14.2	17.2	11.2	15.7	10.2	6.2	8.2	9.0	10.6	11.7	12.4	15.0
27.3.41	4.3	8.5	8.0	3.1	10.6	1.6	7.1	8.2	7.2	6.5	5.2	11.3
28.3.41	1.8	3.1	4.3	5.3	6.1	2.5	3.7	5.2	4.6	5.3	3.7	7.6
29.3.41	1.2	2.3	1.2	3.6	2.4	1.3	4.0	3.0	4.5	3.2	4.9	5.3
30.3.41	1.1	2.5	1.3	4.2	3.7	1.3	2.2	3.6	2.3	1.1	2.6	2.2

TABLE IV.

The hæmoglobin level in per cent of the scorbutic animals and the corresponding animals having the same food-intake but receiving 2 mg. ascorbic acid in addition per animal per day.

No. of animal.	Animals on scorbutic diet.		No. of animal.	Animals on scorbutic diet and ascorbic acid.	
	Initial Hb	Final Hb		Initial Hb	Final Hb
1	86%	68%	1	93%	88%
2	89	72	2	89	86
3	92	70	3	96	92
4	87	69	4	92	83
5	84	65	5	90	91
6	86	68	6	82	80
7	91	73	7	86	86
8	90	71	8	85	86
9	81	71	9	87	85
10	87	69	10	82	83
11	83	65	11	88	89
12	88	68	12	85	83

DISCUSSION

Certain interesting conclusions emerge from the results described in this paper. In the first place it is apparent that with the progress of scurvy there is a progressive diminution in the blood hæmoglobin level of guineapigs, which is almost restored after supplementing the scorbutic diet with ascorbic acid. This lowered hæmoglobin level does not appear to arise merely from a lowered food-intake and therefore a lowered iron-intake, which occurs in scurvy. Tables III and IV indicate that when animals, receiving ascorbic acid, are given restricted amounts of diet equal to those consumed voluntarily by corresponding guineapigs on a scorbutic ration during the previous 24 hours, these animals nevertheless show a normal blood hæmoglobin level. It would thus appear that vitamin C may have some direct rôle in the formation of hæmoglobin. It would appear from the work of Sheet (6) that in nutritional anæmia in man the administration of iron and copper accompanied by ascorbic acid has a pronounced effect on hæmoglobin regeneration. The exact mechanism by which ascorbic acid stimulates hæmoglobin formation requires further work.

Table II and Figures I and II show strikingly that (i) in normal condition the ascorbigen content of the blood of guineapigs is higher than
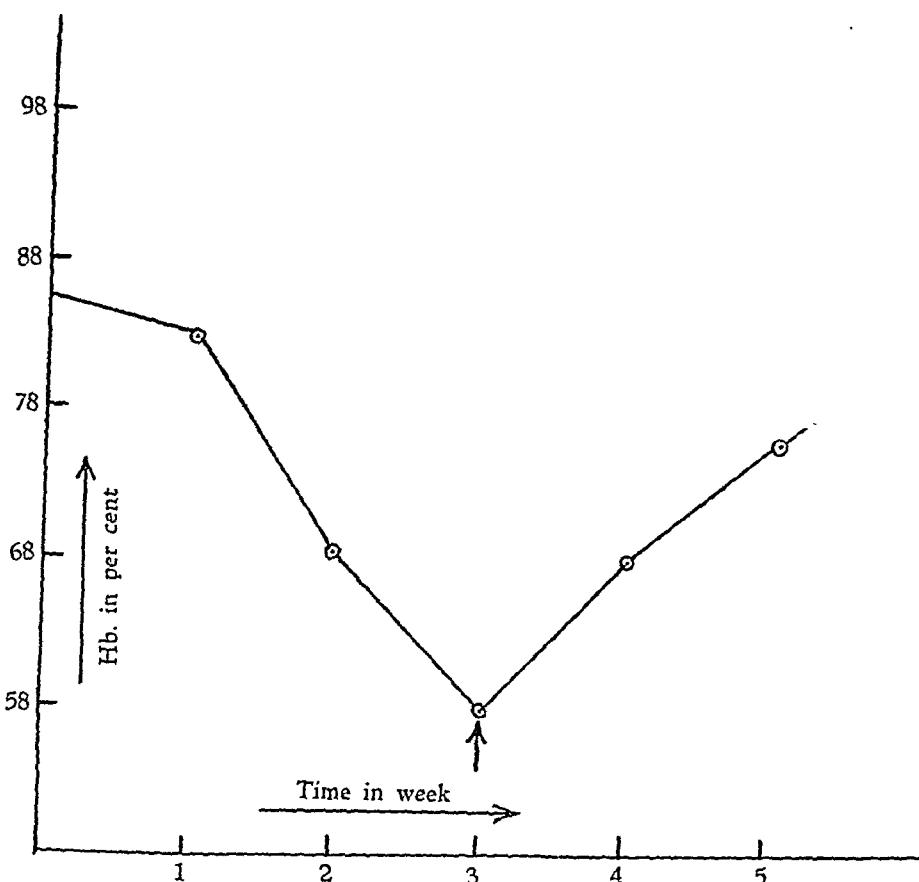
FIG. I. Average blood hæmoglobin concentration of guinea-pigs on a scorbutic diet, supplemented at the arrow mark by 2 mg. of ascorbic acid per animal per day.

the free ascorbic acid content, (ii) with progress of scurvy the levels of both ascorbic acid and ascorbigen in blood undergo progressive diminution, but the latter suffers a more rapid reduction and (iii) with the progressive cure of scurvy by administration of a supplement of ascorbic acid, the ascorbigen level undergoes a more rapid restoration than the ascorbic acid level. These observations make it probable that the ascorbigen of blood serves as a store, from which free ascorbic acid is released for replenishment of the blood and for direct utilisation as and when the body requires it. On a scorbutic diet the body tries, it seems, to keep its blood ascorbic acid level as nearly normal as possible and, therefore, draws upon the blood ascorbigen which is thereby more rapidly diminished than the blood ascorbic acid. During 21 days on a scorbutic diet, for instance, the blood ascorbic acid level ·falls to 50% of its original value, while the blood ascorbigen level

falls to 19% of its original value.   On giving a supplement of ascorbic acid
to scorbutic guineapigs, in the course of 11 days, the blood ascorbic acid
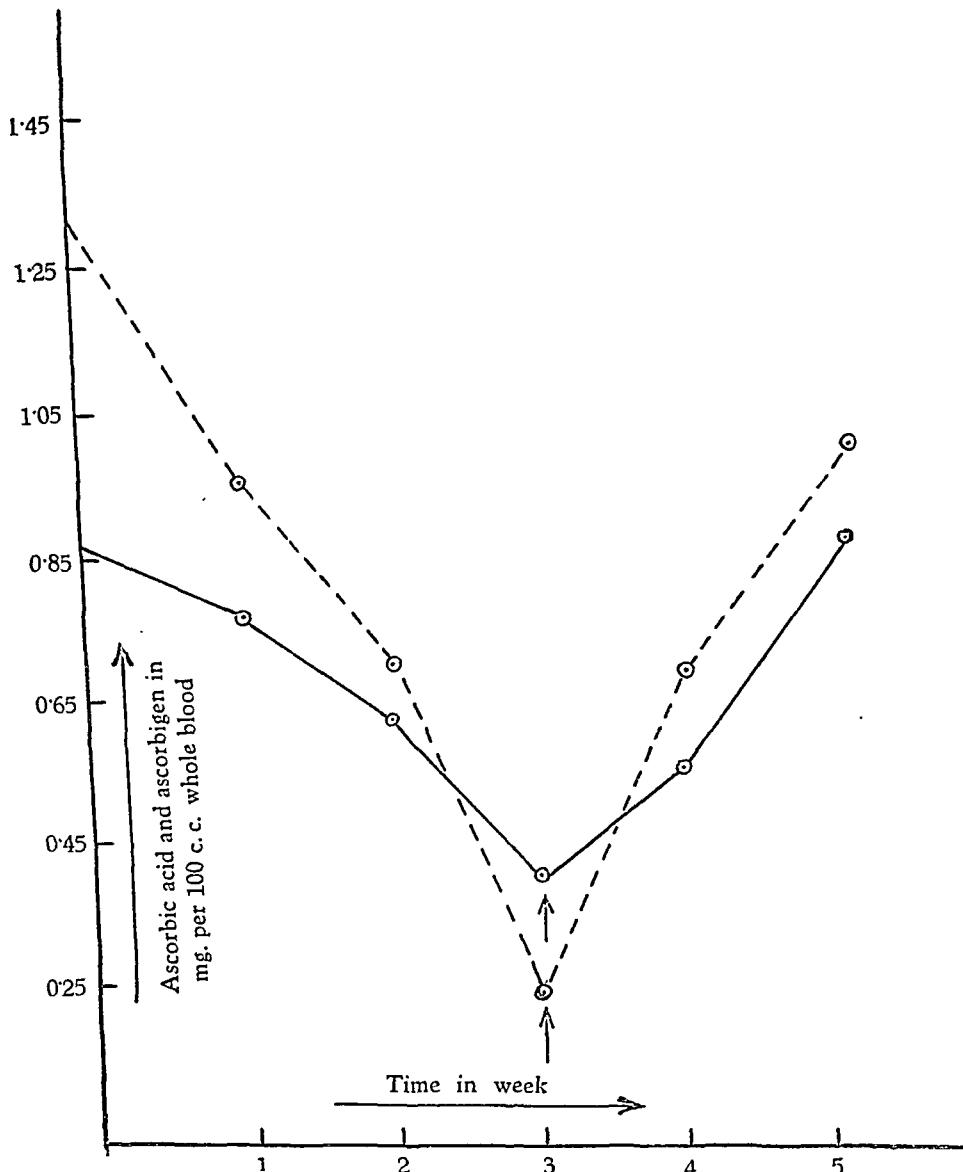
FIG. II.   Average blood ascorbigen and ascorbic acid levels of guinea-pigs on
a scorbutic diet, supplemented at the arrow marks with 2 mg. ascorbic acid per animal
per day.

level is doubled to its normal value while the blood ascorbigen level is
quadrupled, which even then is about 15% below normal.   Blood ascorbigen
therefore seems to play a profound rôle in maintaining the level of blood
ascorbic acid.   Further investigation is proceeding.

SUMMARY

1. The blood ascorbic acid and ascorbigen levels of the blood of guineapigs at different stages of scurvy have been determined. It has been observed that there is a diminution in the concentration of all these substances in blood in scurvy.

2. Administration of ascorbic acid restores the hæmoglobin level. This restoration is not merely due to consumption of more diet and therefore of more iron on the administration of ascorbic acid, as even when guineapigs receiving ascorbic acid are forced to consume restricted amounts of food equal to those taken voluntarily by scorbutic guineapigs, the hæmoglobin level of the blood of these guineapigs remains normal. The vitamin therefore seems to have some more direct relation to the formation of hæmoglobin.

· 3. In normal condition the ascorbigen content of the blood of guineapigs is higher than the free ascorbic acid content. The value of the former is 1.31 mg. and of the latter 0.86 mg. per 100 cc. of blood.

4. With the progress of scurvy blood ascorbigen suffers a more rapid diminution than the free ascorbic acid and with the progressive cure of scurvy by administration of a supplement of ascorbic acid, the ascorbigen level undergoes a more rapid restoration than the ascorbic acid level. The conclusion is drawn that ascorbic acid is stored up in a bound form (as ascorbigen) from which free ascorbic acid is released for direct utilisation as and when the body needs it.

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THE EFFECT OF ORANGE JUICE ON THE ASSIMILATION OF CALCIUM AND THE HISTOLOGICAL STRUCTURE OF THE DEVELOPING TEETH

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It is now recognised that the calcifying dental tissue is very sensitive to changes in calcium metabolism and even slight variations in the calcium and phosphorus content of the diet produce clean-cut responses in the teeth which are not recognizable in other tissues of the body. For sometime past we have been engaged in studying the relative nutritive value of Indian diets whose chief defects are a deficiency of calcium and phosphorus, through their effect upon the developing dental tissues. We have confirmed the view that even slight changes in the calcium and phosphorus content of the food bring about striking changes in the normal appearance of the dentine and the predentine. The formation of the dentine and the predentine is the function of the odontoblasts. The classical work of Höjer (1) showed that one of the characteristic effects of vitamin C deficiency in the guineapig is the degeneration of the odontoblasts. As a result of their critical study on this subject Fish and Harris (2) also concluded that vitamin C was essential for the functional activity of certain types of cells including odontoblasts. Since some of the diets we were studying were also low in vitamin C, we wondered if vitamin C was a factor involved in the abnormal appearance of the dental tissues of our animals. Though it is generally agreed, that vitamin C is not needed by the rat as a species, we decided to test whether the addition of vitamin C to the diet of the rat would make any difference in the structure of the dental tissues. As a source of vitamin C, we fed orange juice and pure ascorbic acid. The results obtained were rather striking and are reported in this paper.

Experimental

A number of young albino rats one month old and weighing between 30 and 40 g., were placed on the following vitamin C deficient basal diet.

Dextrin	...	...	... 63%
Casein	...	. .	... 18%
Dried yeast	...	...	8%
Vegetable fat	...	...	5%
Salt mixture	...	...	5% (McCollum and Davis, 3)
Cod-liver oil ...	...	...	1%

The calcium and phosphorus contents of this diet are 0.4% and 0.47% respectively and Ca:P ratio is 0.85. The diet is complete in all factors known to be essential for the nutrition of the rat.

Two experiments were conducted. In the first preliminary experiment a group of six animals received the basal diet alone and acted as controls, while another group of six rats which were strictly comparable to the first group in age, sex and weight, received 2 cc. of orange juice daily as a supplement to the basal diet. The experiment was continued for 48 days at the end of which the animals were killed for the examination of the histological structure of their teeth.

In the second experiment the animals were divided into three groups of eight. All the animals were one month old and weighed between 30 and 40 g. Each group contained equal number of males and females. Group I received the basal diet alone while the diet of groups II and III was supplemented daily with 2 cc. of orange juice and 1 mg. ascorbic acid in solution at pH 3.0, respectively. This experiment was continued for ·64 days at the end of which the animals were killed for the usual histological study of their teeth.

The details of the technique of the histological examination is fully explained in an earlier paper (Mullick, 4). Histological examination was confined to the upper two incisors. Longitudinal sections of these after decalcification were stained with hæmatoxylin and eosin and the width of the predentine layer near the apical part of the tooth which is stained with eosin was measured.

The results of both these experiments, including the average rate of growth of the animals and the average width of their predentine are shown in Table I.

TABLE I.

Diet.		Number of animals.	Duration of the experiment (days).	Average rate of growth per week (g.)	Average width of the predentine. (μ)
EXPERIMENT I.					
Group I	Basal diet alone.	6	48	6.2	32.0
Group II	Basal diet + 2 cc. orange juice.	6	48	6.1	15.3
EXPERIMENT II.					
Group I	Basal diet alone.	8	63	6.2	30.4
Group II	Basal diet + 2 cc. orange juice.	8	63	8.0	14.2
Group III	Basal diet + 1 mg. ascorbic acid.	8	63	6.6	24.0

DISCUSSION

The value of the histological examination of the teeth in demonstrating the relative nutritive value of the diets has been shown by Mullick and Irving (5), by Gaunt, Irving and Thompson (6), and by Mullick (4). During the normal process of the development of the dentine, calcification follows the secretion of an organic matrix by the odontoblasts. At any given time there is only a narrow band of uncalcified tissues, which constitutes the predentine. But when the calcification does not proceed normally for any reason, the uncalcified layer increases and is often thickened and irregular. We have found that the width of the predentine layer varies with the amount of calcium and phosphorus in the diet, and the various factors which affect the absorption and utilization of these minerals. The authors quoted above have shown that in animals receiving a normal stock diet containing a certain proportion of natural foodstuffs, the width of the predentine layer of the incisors lies between 15 and 20 μ. This figure is increased when there is any abnormality in the calcium and phosphorus metabolism, or a deficiency of these minerals in the diet.

As figures in Table I show, the average width of the predentine of the control animals in both the experiments was found to be 30.4—32.0 μ In view of our experience and of the studies quoted above, this indicated that calcification of the dentine in these animals had not proceeded normally. The feeding of 1 mg. ascorbic acid decreased the average width of the predentine layer to 24.0 while 2 cc. of orange juice brought it down to 14.2—15.3 μ which is the normal figure for a good natural stock diet (see Figure 1). It therefore appeared that with the addition of 2 cc. orange juice to the diet, the normal utilisation of calcium and phosphorus occurred,

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while the effect did not entirely appear to be due to its ascorbic acid content. These results are in conformity with the observation of Lanford (7) who recorded an improvement in the calcium assimilation as a result of orange juice administration. Fig. 1 shows the microphotographs of the longitudinal sections of the three male litter-mates fed on different diets.

The rate of growth was also better in the case of animals receiving orange juice. In experiment II the average rate of growth of the group receiving orange juice was 8.0 g. per week as compared to 6.2—6.6 g. in the case of other groups. In the first experiment, however, difference in growth was not very marked. The factor present in orange juice responsible for better calcification of the teeth is being further investigated.

SUMMARY

1.   The calcium metabolism of groups of rats has been studied by means of a technique based on the examination of the histological structure of the dentine and the predentine of the incisors of the animals.

2.   On a synthetic basal diet in which the calcium utilisation, as revealed by this method, was sub-normal, normal utilisation of this mineral resulted on administration of 2 cc. of orange juice.

3.   The effect of orange juice is not entirely due to its ascorbic acid content as the administration of pure ascorbic acid did not lead to the normal calcification of the dentine.

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FIGURE I

Micro-Photographs of Longitudinal Sections of Upper Incisor Teeth of Male Litter Mates (× 200).

A good stock diet.

Basal diet supplemented with 2 cc. orange juice.

Basal diet alone.
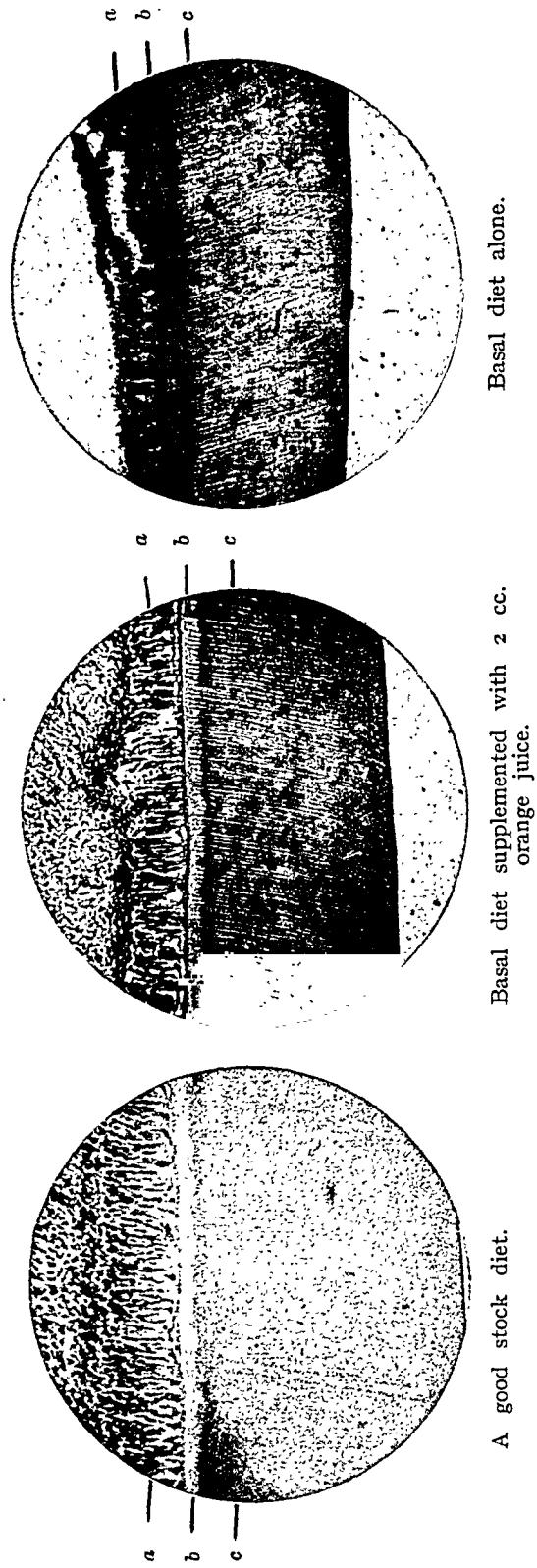
(a) Odontoblasts ; (b) Odontogenic line or predentine : (c) Dentine.

HÆMOPOIETIC EFFECT OF NUCLEIC ACID ON
RATS KEPT ON MILK DIET

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The hæmopoietic action of a considerable number of chemical substances in relation to anæmia has been investigated by various workers. Knowledge, concerning the relationship of vitamin-B complex to hæmopoiesis and anæmia, has been summarized by Sharp (1). It has been shown that in the absence of vitamin-B complex, blood formation may be seriously impaired and anæmia may result. Numerous clinicians have studied the relationship of vitamin B_1 and B_2 to various anæmias and there is some difference of opinion among these investigators.

Guha and Mapson (2) did not regard vitamin B_1 as a hæmopoietic factor, but found vitamin B_2-deficiency to be followed by a diminution in the number of red blood cells and the anæmia was readily cured by autoclaved "Marmite". Copper, glutamic acid and alkaline hæmatin were without effect. It was, however, found that the liver extract in which vitamin B_2 had been destroyed, could nevertheless exert hæmopoietic action in rats, indicating that liver contained a hæmopoietic substance other than vitamin B_2. Subsequently Wills (3) observed that egg-white, as a source of vitamin B_2, could not cure macrocytic anæmia. Guha and Biswas (4) reported, however, that in rats suffering from mild anæmia owing to a vitamin B_2-deficient diet, pure riboflavin had a hæmopoietic effect.

Studies of the possible influences of vitamins on hæmoglobin formation have as yet yielded no conclusive results. It has not been fully established whether any vitamin, which exerts its beneficial effect on blood regeneration does so by direct action on bone-marrow or indirectly by stimulating appetite and intestinal absorption.

The hæmopoietic effect of copper has attracted the attention of a number of investigators during the past few years. Hart *et al* (5) were the first to point out that copper plays a specific rôle in hæmoglobin building and that milk fortified with pure iron salts does not constitute an adequate diet for regeneration of the pigment. Evvard, Nelson and Sewell (6) showed that rats and swine make better gains and exhibit a higher food utilisation

per unit of weight increase when small amounts of copper sulphate are incorporated in the ration. It was observed that in these rats the greater part of the stored copper was confined to the liver. The authors stated that the medicinal and nutritive value of liver and its proper functioning might be somehow related to this element. McHargue, Healy and Hill (7) practically simultaneously with Hart and his co-workers observed that copper stimulates hæmoglobin regeneration of rats kept on an anæmia-producing diet.

Mayers and Beard (8) found that other elements can also increase the speed of regeneration. Particularly they observed that manganese, nickel, zirconium and arsenic have this effect. Titus and others (9) stated that manganese plays a part in synthesis of hæmoglobin. Mitchell and Miller (10) observed that a group of elements is potent for hæmoglobin formation rather than copper alone.

Even a superficial examination of the literature is necessary to reveal that there are considerable divergences of opinion regarding the rôle and necessity of copper in hæmoglobin building. Drabkin and Waggoner (11) stated: "If the milk were copper-free, the problem would be far simpler. One could then definitely assume that copper is a specific supplement to iron in hæmoblogin building. Since, however, anæmia producing milk contains copper and a case of the anæmia has been accomplished by synthetic diets which are lower in copper, in this type of experiment the specificity of copper, or even its necessity, becomes highly questionable."

McCay (12) investigated the influence of some organic factors such as proteins and fats on anæmia of hæmorrhage, and found that rats made anæmic by cardiac bleeding and placed on a diet of lard, sucrose and salt with albumin, casein and liver as three different sources of protein, regenerated more hæmoglobin of liver than on albumin or casein, even though each was supplemented with ferric citrate to make the iron content of the three diets identical.

The foregoing indicates the diversity of food constituents, which apparently play a part in hæmopoiesis. The complexity of the problem is increased because of the different types of natural and experimental anæmias which would conceivably make a difference in requirement of one or other of these accessory factors. It is, for instance, known that certain factors can act as hæmopoietic stimulants in a particular type of anæmia while in the another type they may be ineffective. In the present investigation, we desired to study the factors concerned in hæmopoiesis and erythropoiesis with rats kept on milk as the sole source of diet.

We have been particularly concerned with nucleic acid as a hæmopoietic factor, as we obtained a preparation from fish tissue, which appeared to be iron-nucleotide and it was of interest to study hæmopoietic value of this substance as well as of nucleic acid in nutrition. ·The hæmopoietic effect

of nucleic acid and nucleotides has been studied by several workers. Nucleic acid is an essential constituent of all living cells and is synthesised inside the system and, so far as the present knowledge goes, exogenous supply of this material seems to be unnecessary. Still, it has been observed that if this substance is injected into normal or anæmic vertebrates marked hæmopoiesis results. Karrer *et al* (13) claimed that effective treatment of pernicious anæmia in man was obtained by a single administration of 10—20 mg. of a highly active liver preparation containing apparently an adenine nucleotide, which they considered might be the active agent concerned. Larsell and others (14) observed that washed nuclei from the red blood cells of the fowl, injected into normal rabbits intravenously, produced marked hæmopoietic stimulation. The cytoplasm, from which nuclei have been removed, does not produce hæmopoietic stimulation. Nucleic acid and nucleo-protein obtained from the washed nuclei of the red blood cells of the fowl, injected intravenously into normal rabbits and into anæmic human patients, produce results similar to those resulting from the injection of the nuclei themselves. The investigation of Larsell *et al* (15) concerning the hæmopoietic effect of nuclear extractives in experimental anæmia and in human anæmia showed that nucleoprotein from beef-liver serves as a hæmopoietic stimulation. There is some evidence that nucleoprotein administered with sodium salts of nucleic acid has a more marked effect on hæmopoiesis than either of these substances alone.

The present communication deals with the results of investigations concerning the effect of (1) milk diet, (2) milk diet *plus* iron, (3) milk diet *plus* nucleic acid and (4) milk diet *plus* iron *plus* nucleic acid on the hæmoglobin, erythrocyte and total iron content of rats. The significance of these results in relation to the mechanism by which nucleic acid stimulates hæmopoiesis is discussed.

EXPERIMENTAL

Sixteen adult rats of nearly the same body-weight as far as possible were placed in individual aluminium cages (in order to prevent iron contamination) with screened bottom. Prior to the beginning of the actual experiments they were kept on an exclusive milk diet for 7 days in order to deplete them of any unabsorbed iron. On the 7th day they were divided into 4 groups of 4 animals each. The milk diet was used and continued throughout. Each animal of the first group received daily as supplements 0.5 mg. of iron as ferric chloride, 0.5 cc. of sodium nucleate solution corresponding to 5 mg. nucleic acid and two drops of cod liver oil. The diets of the second and third groups were supplemented with 5.0 mg. of nucleic acid as sodium salt and 0.5 mg. of iron as ferric chloride respectively and all of them received cod liver oil. The fourth group acted as a negative control on milk diet *plus* cod liver oil.

Before giving the supplement to the animals, the blood hæmoglobin and erythrocyte contents were determined by the Shalli's hæmatometer and by the total count method respectively. The blood was drawn out by clipping off the end of the tail. These determinations of each animal were made weekly and the experiments were continued for 5 weeks after which the animals were again kept on milk diet for 4 days in order to deplete them of any unabsorbed iron. They were killed, dried at 105° for 24 hours and ignited to a white ash in a silica crucible for the determination of the total iron of the body. The total iron content was determined by the thiocyanate method as used by Saha and Guha (16).

The hæmoglobin and erythrocyte responses are shown in Figures 1 and 2 and the total iron content in Table I. Each curve in Figures 1 and 2 represents the average of results of the four animals of each group.

TABLE I.

Iron content of the animal body after feeding them with supplements of nucleic acid and iron.

Group and supplement.	Initial body wts.	Final body wts.	Increase in body wts.	Mean increase in body wts.	Iron content per 100 g. of body wts.	Mean iron content per 100 g. of body wt.
Group I	55 g.	87 g.	32 g.		4.8 mg.	
(5 mg. nucleic	49	77	27	32.2 g.	5.2	5.1 mg.
acid + 0.5 mg.	58	100	42		4.6	
iron)	47	79	32		5.6	
Group II	50	87	37		1.9	
(5 mg. nucleic	57	88	31	34.2	3.2	2.5
acid)	57	100	43		2.0	
	42	68	26		3.0	
Group III	41	76	35		8.6	
(0.5 mg. iron)	40	71	31	32.7	10.3	9.0
	46	89	43		7.9	
	48	70	22		9.2	
Group IV	49	81	32		3.9	
(no supplement)	46	80	34	34.0	4.0	4.0
	43	73	30		3.8	
	46	86	40		4.1	

DISCUSSION

From the above figures and table, it is clear that both the hæmoglobin and erythrocyte formations in the rat are greatly enhanced by supplementing iron and nucleic acid to a basal milk diet. The total iron content of the body in this group of animals seems to be slightly higher than what has been reported by Shythe and Miller (17) for animals of the same age and body-weight. They stated that the amount of iron in the rat body which is highest at birth, *viz.* 0.0055% of the body-weight, drops to 0.0026% after 20 days and then rises to 0.0043% after 100 days and for adult rats on a commercial calf-meat diet the iron content averages about 0.0051%. The iron values obtained with the calf-meat diet agrees fairly with our findings, probably due to the fact that calf-meat contains a high percentage of nucleic acid which makes the iron more available than the ordinary normal diet.

On administering nucleic acid alone, stimulation of the hæmopoiesis results upto the second week, after which both the blood hæmoglobin and erythrocyte count fall abruptly to a low value and then upto the 5th week the hæmoglobin content falls more slowly but the erythrocyte count maintains almost a steady value. In this group of animals the total iron content of the rats falls below the normal. These results indicate that so long as the stored iron of the body can supply the need, its mobilisation for the purpose of hæmopoiesis is stimulated by nucleic acid, and then due to the shortage of stored iron both the hæmoglobin and R.B.C. contents fall abruptly to a low value. It has also been found that the blood coagulation time increases considerably after the expiry of the second week. It would thus appear that, so long as the body-store of iron is sufficient, nucleic acid supplement would stimulate hæmopoiesis and erythropoiesis, but when the store is very much reduced the effect of nucleic acid may be deleterious. It would follow that in those anæmias where tissue iron is greatly depleted, nucleic acid unsupplemented by iron may have indeed an injurious effect. On the other hand, administration of nucleic acid *plus* iron appears to supplement milk effectively for hæmopoiesis and erythropoiesis. It is likely that these supplements would have similar action in both normal and anæmic cases.

From Figures 1 and 2 and from Table I it is interesting to note that the addition of iron to milk diet has no effect on the hæmoglobin or erythrocyte content of the blood but increases the total iron of the body to a great extent. These findings are in close agreement with those of Elvehjem and Sherman (18) which indicate that addition of pure iron to the milk diet of anæmic rats, which had been well depleted in their reserve of iron had no effect on the hæmoglobin content of the blood, but increase the total iron content of the liver and spleen to a large extent. It would appear therefore that milk cannot be supplemented effectively for hæmopoiesis by iron alone.

FIG. 1

FIG. 2

Time in week
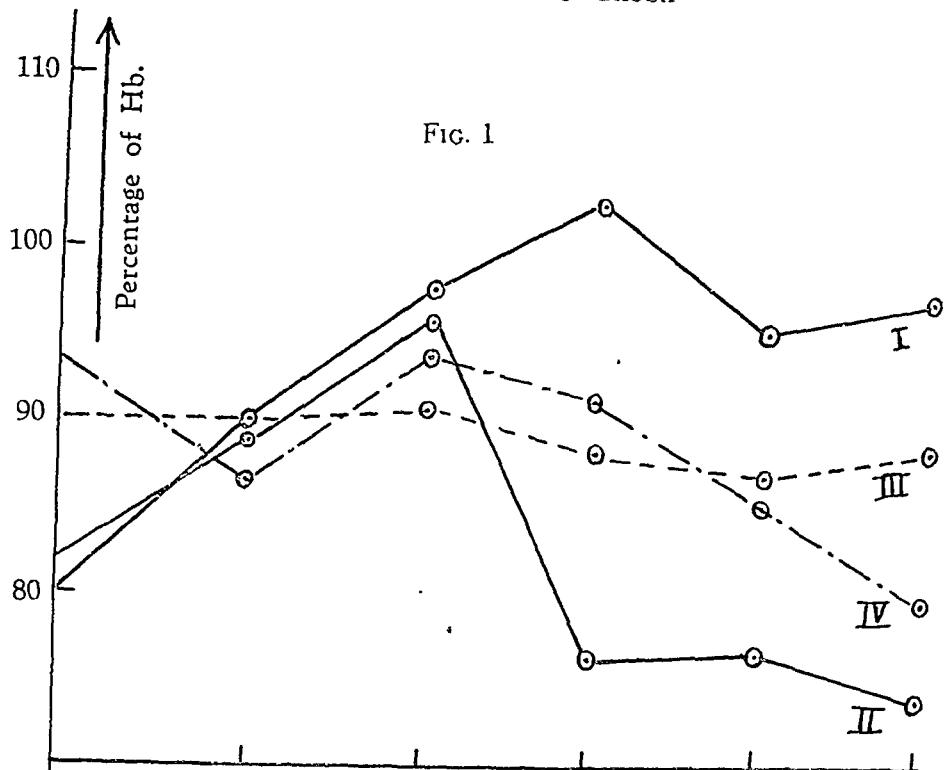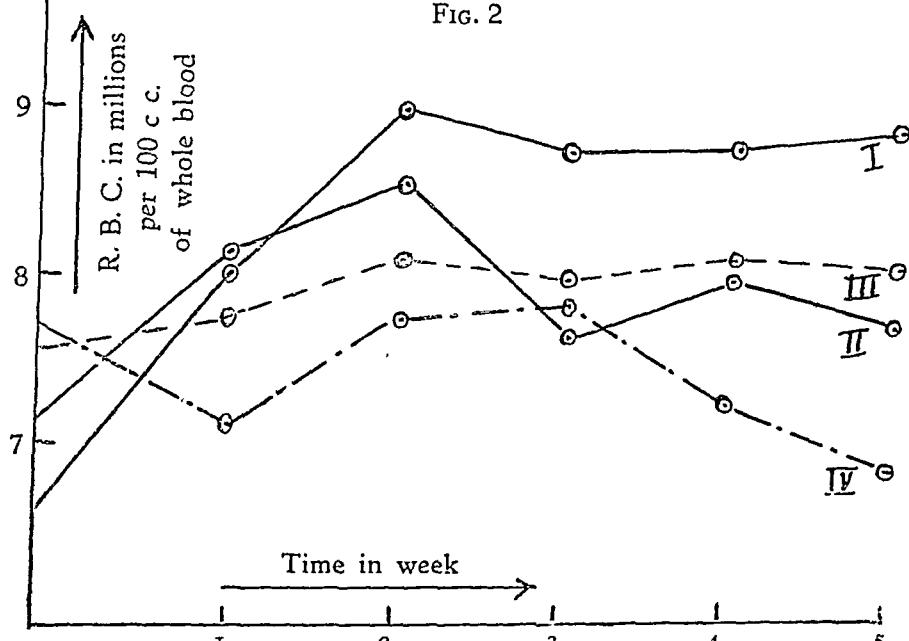
I.—Milk diet supplemented with 0.5 mg. of iron as $FeCl^3$ and 5 mg. of nucleic acid.
II.—Milk diet supplemented with 5 mg. of nucleic acid.
III.—Milk diet supplemented with 0.5 mg. of iron.
IV.—Milk diet only.

When the rats are kept on milk as the sole source of diet, the hæmoglobin and R.B.C. content of the blood maintained almost a steady level upto the 3rd week of experiment, probably at the expense of the stored body iron and when the reserve iron reaches limiting value, the above values begin to decrease slowly. The total body iron falls below the normal level. No sign of definite anæmia (a value of hæmoglobin below 70 per cent) develops, however, even in the 5th week.

As all these experiments were carried out in the animals, which were on milk diet for 5 weeks and had not developed anæmia, it is clear that the hæmopoietic and erythropoietic action of nucleic acid *plus* iron is effective under normal psysiological condition. It is probable that it is also effective at least in some types of anæmic conditions.

Addition of nucleic acid to milk diet does not however promote growth which is clear from the fact that the mean increment of body-weights is the same in all cases.

SUMMARY

1. Blood hæmoglobin and erythrocyte are markedly enhanced by supplementing the milk diet of rats with iron and nucleic acid. The total iron content of the rats also increases appreciably.

2. On supplementing the milk diet with nucleic acid alone, stimulation of hæmopoiesis results upto the second week after which the blood hæmoglobin and R.B.C. fall to a low value. The stored iron of animals is greatly decreased.

3. If the milk diet is supplemented with iron alone hæmopoiesis is not stimulated but the reserve iron is greatly increased.

4. When the rats are kept on milk diet, the blood hæmoglobin and erythrocyte levels maintain a steady value upto the third week, after which they begin to decrease slowly.

5. Nucleic acid has no growth-promoting value.

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NUTRITIONAL INVESTIGATIONS ON FISH

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In our previous publications (1, 2, 3), the results of analyses of 37 different varieties of Bengal fresh-water fish with reference to water, body fat, protein, calcium, phosphorus, total iron, ionizable iron and copper have been reported. The present communication deals with the results of 11 more varieties of fish of which 4 varieties have been obtained from the Bay of Bengal. As certain varieties of fish are not available throughout the year, it is necessary to carry on the investigation from season to season. The seasonal variations of the same species of fish also require investigation.

The percentage quantities of water, body fat, protein, ash, total and ionizable iron, calcium, phosphorus, and copper in 11 different varieties of fish have been estimated. From the results obtained it is found that among the samples analysed, Parsey (*Mugil parsia*) has the highest fat content, 4.96%, and then come Chela (*Chela bacaila*) and Kolshe (*Colisa fasciata*), 4.3% and 3.85% respectively. Mahasole (*Barbus (Tor) tor*) containing 25.2% protein has been found to be the richest in protein among all the varieties of fish which have been hitherto investigated. As regards the calcium content, Bata (*Labeo bata*) had the highest 0.79%. The next good sources of calcium are Parsey (*Mugil parsia*) and Chela (*Chela bacaila*). The phosphorus content is found to be highest in Kholshe (*Colisa fasciata*), 0.36%, after which comes Gurjowlli, 0.30%. The fish Chela has an unusually high content of copper, 0.51 mg. per cent. The four species of seafish investigated did not have any special features, distinguishing them from the fresh-water fish.

EXPERIMENTAL

The methods employed in these investigations are the same as described in the previous papers, except in the case of ionizable iron, which has been estimated by the technique described by Saha and Guha (4). This method

TABLE I.

Mean values of water, body fat, ash and protein per 100 g. of fish muscle tissue.

Season of collection.	Bengali name.	Zoological name.	Ranges of body wts. (g.)	Water (g.)	Body fat (g.)	Ash (g.)	Protein (g.)	Average percentage accounted for	Remarks.
January, 1941	*Lady vendi	Sillags sihasna	131—170	77.0	0.67	0.82	18.2	96.7	
,,	*Palmplate	Pampus argenteus	48—91	81.0	0.97	0.71	15.1	97.8	
,,	Chela	Chela bacaila	3—11	77.5	4.30	2.10	14.6	98.5	The whole fish was taken.
,,	*Amlet	...	250—430	73.5	1.09	0.91	20.8	96.3	
,,	Kholshe	Colisa fasciati	7—19	75.0	3.85	1.85	16.1	96.8	The whole fish was taken.
February, 1941	Bhangar	Mugil tade	117—201	76.3	0.57	1.21	18.9	97.0	
,,	Bata (small varieties).	Labeo bata	5—25	79.0	2.48	2.00	14.3	97.8	
,,	*Parsey (big varieties).	Mugil parsia	180—250	72.3	4.96	0.86	18.4	96.5	
February 1941	Chital	Notopterus chitala	5—14 (in lbs.)	75.0	2.32	1.01	18.6	96.9	
,,	Mahasole	Barbus (Tor) tor	36—55 (in lbs.)	70.3	2.26	1.20	25.2	99.0	
November, 1940	Gurjowli		236—807	81.0	1.10	1.26	16.1	99.4	

TABLE II.

Mean values of calcium, phosphorus, total iron, ionizable iron and copper per 100 g. of fish muscle tissue.

Bengali name.	Zoological name.	Calcium (g.)	Phosphorus (g.)	Total iron (mg.)	Ionizable iron (mg.)	Copper (mg.)
*Lady vendi	*Sillags sihansa*	0.26	0.20	0.73	0.60	0.23
*Palmplate	*Pampus argenteus*	0.24	0.14	1.64	0.83	0.12
Chela	*Chela bacaila*	0.59	0.34	1.96	0.96	0.51
*Amlet	...	0.26	0.22	1.11	0.45	0.143
Kholshe	*Colisa fasciata*	0.46	0.36	0.90	0.65	0.10
Bhangar	*Mugil tade*	0.31	0.14	0.01	0.31	0.15
Bata (small varieties)	*Labeo bata*	0.79	0.20	1.09	0.01	0.17
*Parsey (big varieties)	*Mugil parsia*	0.65	0.28	3.27	0.60	0.14
Chital	*Notopterus chitala*	0.18	0.25	2.98	0.50	0.165
Mahasole	*Barbus (Tor) tor*	0.13	0.28	3.83	0.80	0.122
Gurjowli		0.38	0.30	1.26	0.56	0.10

involves the treatment of the fish tissue with 10% acetic acid, followed by reduction with sodium hydrosulphite and treatment with $\alpha\alpha'$ -dipyridyl, which has been found to give a higher values for available iron than that obtained by Hill's method.

The mean values, given in Tables I and II, have been obtained from analyses of 4 to 5 samples of each variety of fish except in the case of Mahasole (*Barbus (Tor) tor*) of which only two samples were available. Those fish that are marked with an asterisk are sea-fish derived from the Bay of Bengal.

Our best thanks are due to Dr. Baini Prashad and Dr. H. L. Hora of the Zoological Survey of India for supplying the scientific names of some of the fish and also to the Indian Research Fund Association for a research grant.   Our thanks are also due to Dr. B. C. Guha for his advice.

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CONSTITUENTS OF *PACHYRRHIZUS ANGULATUS*, RICH

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Pachyrrhizus angulatus, Rich (=*P. trilobus, Linn.* =*Dolichos bulbusus,*
D.C.) is known as Yaka, or Wayka in English, Sák álú in Bengali and
Chána álú in Hindi. It is a robust climbing plant cultivated in moist lands.
In India is is mainly localised to Bengal. The plant is not known in the
wild state. The tuberous roots are spindle shaped and are usually below 8″
in length and slightly smaller in diameter. The tubers are usually eaten raw.
They are sweet and resemble turnip in consistancy. When boiled they are
dirty grey in colour and insipid in flavour but are considerably used in
times of scarcity (1). There are two abnormally big tubers in the food gallery
of Indian Museum, Calcutta, weighing about 16 lbs. each and having a
diameter of about 10″. They are the biggest tubers yet recorded. The tubers
available in Calcutta markets are of all sizes but usually below 5″ in diameter.
They can be stored for a long period without much deterioration. It is
one of the important vegetables consumed without cooking.

Average length of the root analysed was 3″ and the diameter 2″. The
edible portion was analysed after removing the fibrous skin. Two varieties
(possibly due to different age of the tuber) were noticed, the one being un-
palatable and of very tough consistancy and apparently more fibrous and
the other being softer and sweeter. Only the sweeter variety was analysed.
The sample were purchased locally.

EXPERIMENTAL

Moisture was determined by heating the representative samples in steam-
oven and desiccating them to a constant weight in vacuum desiccator. The
fresh plant material was prepared for determination of soluble reducing and
non-reducing sugars. It was extracted in a Soxhlet apparatus with 80%
alcohol until all soluble sugars were extracted. The alcohol was distilled off
and the residue was completely dried, and dissolved in water. Reducing

sugar was determined in an aliquot portion ; non-reducing sugar was determined after hydrolysing with dilute hydrochloric acid.   The starch was determined with Taka-diastase by hydrolysing the residue left after extraction of soluble sugars (2, 3).   Protein was determined by Kjeldahl method. Lipoids were estimated by extracting large quantities of desiccated and powdered tubers in Soxhlet extractor with ether.   Ash was obtained by incinerating the dry material in muffle furnace below 45°.   The following table gives the composition of tubers.

| Moisture | ... | ... | ... | ... | 82.38 per cent. |

| Starch | ... | ... | ... | ... | 9.72 ,, ,, |

| Reducing sugars (expressed as glucose) | | | | ... | 2.17 ,, ,, |

| Non-reducing sugars (expressed as glucose) | | | | ... | 3.03 ,, ,, |

| Protein | ... | ... | ... | ... | 1.47 ,, ,, |

| Lipoids | ... | ... | ... | ... | 0.09 ,, ,, |

| Ash | ... | ... | ... | ... | 0.50 ,, ,, |

	Copper (4)	0.43 mg. per 100 g. of tuber.
	Iron (5)	1.13 ,, ,, ,,
	Calcium (6)	16.00 ,, ,, ,,

| Fibre etc. | ... | ... | ... | ... | 0.64 ,, ,, |

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STUDIES ON VITAMINISED OIL.   PART I.

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Milk fat (butter) has all along been considered to be of unique nutritive value, and it is now known that its speciality lies in its richness in absorbable fatty glycerides and the essential vitamins, particularly vitamin A.   For the latter, cod liver oil is being largely used ; but its highly unsaturated $C_{20} - C_{22}$ glycerides have often been found (1, 2, 3) to exert a harmful action on the animal system when administered over long periods of time.   Further, the vitamin A of this marine oil undergoes oxidation on storage (4, 5).   From economic and commercial points of view the incorporation of some vitamin concentrate in suitable vegetable oil is now being advocated (*British Pharmacopœia,* 1932).   Two questions, however, arise and these are: whether it is advisable to take a fat such as arachis oil rich in long-chain fatty acids as these are not so readily assimilated by the body.   But Barbour (6), from his feeding experiments on rats, did not find any accumulation of fat from the arachis oil in the liver ; and the arachidic acid present was almost quantitatively excreted.   The second point is that being composed of a vegetable fat the vitaminised oil may also develop rancidity due to the formation of peroxides by the action of air on the unsaturated glycerides present, and the peroxides thus formed, may destroy the added vitamin potency.   It was, accordingly, considered to be of interest to undertake an investigation on the relative stability of such an oil prepared from arachis and the commonly used olive oil when stored under customary conditions.   The work is in progress ; but in the mean time the stability has been studied by passing dry, carbon-dioxide free air through the different vitaminised oils and cod liver oil.   The results so far obtained are recorded below.

EXPERIMENTAL

Cod Liver Oil—A pure genuine cod liver oil, C.-P. value 9, was taken from the stock of Bengal Immunity Co., Ltd., and was subjected to aeration in a glass vessel closed with a velvet cork with two borings for holding the inlet and outlet tubes.   Air, previously purified by passing through concentrated sulphuric acid and soda lime, was sucked through the oil placed in the vessel at room temperature (27-30°).   From time to time the vitamin A content was determined by the Carr-Price method.   The peroxide value (in

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terms of cc. of 0.01-N iodine) was also simultaneously ascertained by the method of Nakamura (7) as modified by Basu and Mazumder (8). As the peroxides, the first products of oxidation of unsaturated fats, undergo subsequent disruption to aldehydic compounds with ultimate formation of acids, the changes in the concentrations of these two constituents during the progress of oxidation were also recorded. The aldehyde value (in terms of cc. of 0.002-N iodine) was determined by extracting the product with sodium bisulphite solution in absence of light (cf., Basu and Mazumder, 8).

Vitaminised Oils—(a) Arachis oil used had the following specifications: specific gravity at 31°, 0.91 ; refractive index at 31°, 1.466 ; acid value, 0.44 ; iodine number, 91.66); saponification value, 185.9 and unsaponifiable matter, 0.985 per cent. (b) Olive oil had the following characteristics: Specific gravity at 31°, 0.9117 ; refractive index at 31°, 1.4659 ; acid value, 0.25 ; iodine number, 83.6 ; saponification value, 181.0 ; and unsaponifiable matter, 1.0 per cent.

Both the oils were mixed with concentrates of vitamins A and D secured from Bengal Immunity stock in such a way that the finished products contained 1000 international units each of the two vitamins per g. as specified in the *Second Addendum of the British Pharmacopœia* (1932).

· The "Oleum Vitaminatum" (B.P.) thus prepared from arachis as well as olive oil was subjected to aerial oxidation.. The results of all the experiments are given in Table I.

TABLE I.

Stability of Vitamin A in various oils.

Oxidation period in hours.	C.-P. Unit			Peroxide value			Aldehyde value			Acid value		
	Cd	Vo	Va	Cd	Vo	Va	Cd	Vo	Va	Cd	Vo	Va
0	9	9.5	10	3.1	1.3	0.2	0.9	2.2	0.26	1.0	0.6	0.42
20	9.5	10.2	11	5.4	2.3	2.1	—	—	—	—	—	—
40	9.5	6.3	10.2	7.6	2.5	3.1	—	—	—	—	—	—
60	6.3	2.4	9.5	14.0	3.8	3.4	—	—	—	—	—	—
70	4.35	1.7	—	40.0	8.0	—	—	12.5	—	—	1.5	—
80	2.0	—	—	70.0	—	—	—	—	—	—	—	—
90	0.2	—	8.7	150.0	—	4.2	?	—	—	7.9	—	—
120	—	—	7.9	—	—	4.6	—	—	—	—	—	—
150	—	—	5.8	—	—	4.9	—	—	—	—	—	—
180	—	—	4.0	—	—	5.8	—	—	3.7	—	—	0.8

Cd = Cod liver oil ; Vo = Oleum vitaminatum (B.P.) from olive oil ; Va = Oleum vitaminatum (B.P.) from arachis oil.

? — The aldehydic value could not be determined on account of stable emulsion formation.

It is a general belief that the organic peroxide, formed during the oxidation of any fat, is responsible for the destruction of vitamin A. It would therefore be not unreasonable to expect that the addition of a suitable anti-oxidant which is known to prevent the oxidation of fat (*cf.* Mouren, 9) would also stabilise the vitamin present in the oil. Accordingly, the Oleum Vitaminatum from arachis oil was mixed with hydroquinone (0.2 per cent) (Jones and Christiansen, 10) and air was drawn through it as usual. The experimental observations are recorded in Table II.

TABLE II.

Stability of vitaminised arachis oil incorporated with hydroquinone.

Oxidation period in hours.	C.-P. unit.	Peroxide value.	Aldehyde value.	Acid value.
0	10	0.8	0.25	0.4
60	9.0	1.0	—	—
120	9.0	1.15	—	—
180	9.0	1 0	—	—
300	8.95	1.55	1.46	0.51

DISCUSSION

From the experimental data so far obtained it is evident that with the increase of peroxide value, the rate of destruction of vitamin A increases. Amongst the three products studied the vitaminised arachis oil is least susceptible to oxidation (*vide* Table I). The antioxidant, hydroquinone (0.2%) renders the vitaminised oil resistant to oxidation. Thus in Table II it may be noticed that though there was a loss of 10 per cent potency during first 120 hours' aeration, the oleum maintained about 30 per cent of its vitamin A activity even when air was passed for 300 hours. The increases in peroxide, aldehyde and acid values were also not so marked in presence of hydroquinone.

The presence of free fatty acid often conduces to the onset of oxidation, but none of the oils used was rich in its acid content (*vide* Table I). It, however, increased with the progress of oxidation. This increase is no doubt due to the disruption of the initial products of oxidation, the peroxides. The question, therefore, is whether the destruction of vitamin A is solely dependent on the formation of peroxides in fats or oils (*cf.*, Smith, 11). From the data so far recorded it is difficult to draw such a conclusion. The characteristics found in the "oleum vitaminatum" from olive oil, on the contrary, tend to show that there might be some other factor or factors responsible for the decrease of vitamin potency. The vitamin A content of the vitaminised arachis oil should have diminished more rapidly as arachis oil is richer in unsaturated glycerides (dioleolinoleins) than olive oil.

Apparently the stability of the vitaminised oil depends on the nature of the unsaturated glycerides present in the oil. Olive oil contains about 45 per cent triolein whereas it is absent in arachis oil (Hilditch, 12) ; the latter also contains a fairly high percentage of linoleic acid which is considered essential for fat deficiency disease (Burr and Burr, 13-14 ; Williams and Andenon, 15). In fact, a vitaminised oil prepared from arachis oil base that is being clinically tested elsewhere under the proprietory name of "Bi-Adol", is being found to be well-absorbed without causing any gastric or other physiological disturbances.

To have a more definite knowledge about the stability of vitaminised oils, it would, however, be necessary to study vitamin-liquid paraffin systems containing individual glycerides.

SUMMARY

1. Aerial oxidation reduces the vitamin A-potency of vitaminised oils.

2. Oleum vitaminatum prepared from arachis oil when incorporated with 0.2% hydroquinone retains its potency to a considerable extent.

3. The problem of destruction of vitamin A in oils has been discussed.

In conclusion the author wishes to express his sincere thanks to Mr. J. C. Sen Gupta for helping him in certain experimental portions of the work.

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NEUTRALISATION OF *VIPERA RUSSELLII* VENOM BY THE HOMOLOGOUS ANTI-SERUM

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(Received for publication, April 23, 1941)

Recently Banic and Ljubetic (1) have described in detail a method for the assay of anti-serum prepared against the venom of *Vipera ammodytes*. They have determined the doses of serum required to neutralise the venom at different levels by intravenous injection into mice and have found that the volume of serum neutralising 2 d.c.l. (dose certainly lethal) is much greater than twice the volume required to neutralise 1 d.c.l. The authors explain the result on the assumption that the greater part of 1 d.c.l. is rendered ineffective by the natural resistance of the experimental animal, and only a small part, about one-fifth of 1 d.c.l., is left over to be neutralised by the anti-serum for ensuring protection. They further reported that neutralisation of the venom of *Vipera ammodytes* by the homologous anti-serum can be represented by the equation:

$$y = (x-1)a + s_1 \qquad (i)$$

where y and s_1 denote the cubic centimeters of the anti-serum required to neutralise x d.c.l. and one d.c.l. of venom respectively ; and a is a constant, characteristic of the sample of the anti-serum.

Anti-sera against the venom of *Vipera russellii* are now manufactured in a number of institutions in India and an investigation on the neutralisation of the *V. russellii* venom by its antivenene was undertaken with the object of ascertaining if the method of assay developed by Banic and Ljubetic could be applied to this case also.

EXPERIMENTAL

Anderson (2) reported that the m.l.d. of the *Vipera russellii* venom could be ascertained accurately only when injections are given by the intravenous route. In our attempt to determine the d.c.l., we tried injections of the venom by the subcutaneous, the intramuscular and the intravenous routes, and obtained consistent results, only when the route of injection was intravenous. In our experiments, pigeons, weighing between 290 and 300 gms. were used. For each experiment 5 animals were used and the dose of venom which killed all the five injected animals within 15 minutes was taken as one d.c.l. The d.c.l. of the sample of venom used by us was found to be 0.015 mg.

NEUTRALISATION TESTS AT DIFFERENT LEVELS

The doses of serum (A) required to neutralise various multiples of 1 d.c.l. of venom were determined. As described above pigeons were used as experimental animals and injections were given by the intravenous route. Solutions of the venom and the anti-serum-A were mixed in different proportions and incubated at 30° for half an hour before they were injected. Five pigeons were used for each set of experiments. The results are recorded in Table I. In Table II, the experimentally determined values of the doses of serum neutralising different multiples of 1 d.c.l. of the venom are compared with those calculated from equation (i). The value of a in equation (i) was calculated from the equation,

$$y_1 - y_2 = (x_1 - x_2)a \qquad (ii)$$

by substituting in it, the experimentally determined values of y_1 and y_2, corresponding to known values of x_1 and x_2 respectively. The equation (ii) follows directly from the equation (i). It will also be noticed that

$$1/a = \frac{(x_1 - x_2)}{(y_1 - y_2)} = \text{the number of d.c.l. of venom which 1 cc. of the}$$

serum can neutralise and thus gives a measure of the neutralising power of the serum. It will be noticed from the data, recorded in Table II that the values of the neutralising doses of the anti-serum, calculated from equation (i), agree well with those determined experimentally up to the level of 20 d.c.l. of venom. When the dose of venom was higher than 20 d.c.l., the observed values were much greater than those calculated from the equation.

TABLE I.

SERUM A.

Venom used.	No. of d.c.l.	Serum added.	Mortality ratio.	Neutralising dose of serum.
0.015 mg.	1	0.0005 cc.	5/5	
0.015	1	0.00075	5/5	
0.015	1	0 001	0/5	0.001 cc.
0.075 mg.	5	0.020 cc.	5/5	
0.075	5	0.021	4/5	
0.075	5	0.022	0/5	0.022 cc.
0.15 mg.	10	0.03 cc.	5/5	
0.15	10	0.04	5/5	
0.15	10	0.045	4/5	
0.15	10	0.050	0/5	0.05 cc.
0.30 mg.	20	0.10 cc.	5/5	
0.30	20	0.103	3/5	
0.30	20	0.105	0/5	0.105 cc.
0.45 mg.	30	0.22 cc.	5/5	
0.45	30	0.23	4/5	
0.45	30	0.24	0/5	0.24 cc.

TABLE II.

$a = 0.0053.$

No. of d.c.l. of venom used. (1)	Neutralising dose of serum (A).		Difference between (2) and (3)
	Observed. (2)	Calculated from equation (i). (3)	
1	0.001 cc.	—	—
5	0.022	0.022 cc.	0.000 cc.
10	0.050	0.049	0.001
20	0.105	0.102	0.003
30	0.240	0.155	0.085

EFFECT OF DILUTION ON THE POTENCY OF THE ANTI-SERUM

The influence of dilution on the neutralising power of the serum (A) was also studied. Two samples (B) and (C) were prepared by diluting (A) two and four times respectively of its original volume with physiological saline. Calculated on the basis that potency varies inversely as the dilution, the neutralising power per cc. of (B) and (C) should be half and one-fourth respectively of that of 1 cc. of (A). The doses of (B) neutralising various multiples of 1 d.c.l. of venom were ascertained and are recorded in Tables III and IV. It will be noticed from the data in Table IV that the observed values of the neutralising dose agree well with those calculated from equation (i), up to the level of 25 d.c.l. Beyond this level the potency of the serum determined experimentally is much less than that calculated from the equation of Banic and Ljubetic. It follows, therefore, that the constancy of the neutralising power as assumed by Banic and Ljubetic does not hold good in the case of the anti-serum against the *Vipera russellii* venom, when the level of the venom dose exceeds a certain limit.

In Table V, the doses of serum (C) neutralising ten and twenty d.c.l. of venom are recorded. In Table VI, the number of d.c.l. of venom neutralised by 1 cc. of the sera (A), (B) and (C) respectively are recorded along with those calculated on the basis that the potency of the anti-serum varies inversely as the dilution ratio. It will be noticed that the two sets of values agree quite well. This shows that within the range investigated dilution of the anti-serum does not cause any alteration in the activity of the antibody molecules or micelles.

TABLE III.
SERUM B.

Venom used.	No. of d.c.l. used.	Serum added.	Mortality ratio.	Neutralising dose of serum.
0.015 mg.	1	0.00075 cc.	5/5	
0.015	1	0.00010	5/5	
0.015	1	0.0015	5/5	
0.015	1	0.002	0/5	0.0020 cc.
0.075 mg.	5	0.03 cc.	5/5	
0.075	5	0.035	5/5	
0.075	5	0.040	4/5	
0.075	5	0.045	0/5	0.045 cc.
0.15 mg.	10	0.08 cc.	5/5	
0.15	10	0.09	5/5	
0.15	10	0.01	3/5	
0.15	10	0.0105	0/5	0.0105 cc.
0.30 mg.	20	0.20 cc.	5/5	.
0.30	20	0.21	5/5	
0.30	20	0.215	3/5	
0.30	20	0.22	0/5	0.22 cc.
0.375 mg.	25	0.25 cc.	5/5	
0.375	25	0.26	5/5	
0.375	25	0.265	2/5	
0.375	25	0.27	0/5	0.27 cc.
0.45 mg.	30	0.40 cc.	5/5	
0.45	30	0.42	5/5	
0.45	30	0.44	2/5	
0.45	30	0.45	0/5	0.45 cc.

TABLE IV.

$a = 0.0106.$

No. of d.c.l. of venom used. (1)	Neutralising dose of serum (B).		Difference between (2) and (3)
	Observed. (2)	Calculated from equation (i). (3)	
1	0.002 cc.	—	—
5	0.045	0.0439 cc.	0.0011 cc.
10	0.105	0.097	0.008
20	0.22	0.203	0.017
25	0.27	0.256	0.014
30	0.45	0.309	0.139

TABLE V.,

SERUM C·

Venom used.		No. of d.c.l.	Serum added.		Mortality ratio.	Neutralising dose.
0.15	mg.	10	0.170	cc.	5/5	
0.15		10	0.185		4/5	
0.15		10	0.200		0/5	0.200 cc.
0.30	mg.	20	0.410	cc.	5/5	
0.30		20	0.420		5/5	
0.30		20	0.425		4/5	
0.30		20	0.430		0/5	0.430 cc.

TABLE VI.

Sample of serum used.	No. of d.c.l. neutralised per cc.	
	Observed.	Calculated from the dilution ratio.
A	188	—
B	94	94
C	44	47

DISCUSSION

It will be noticed from the data recorded in Tables I and III that the quantity of serum required to neutralise 5 d.c.l. is much greater than 5 times the quantity which neutralises 1 d.c.l. This is in agreement with the observation of Banic and Ljubetic. The linear relationship between the doses of venom of the *Vipera ammodytes* and the corresponding neutralising doses of the anti-serum, noticed by Banic and Ljubetic, holds in the cases of *Vipera russellii* venom and its antivenene for a short range—from 1 to 20 d.c.l. only. It is within this range therefore, that determination of potency of the anti-sera against the venom of *Vipera russellii* should be made in order to obtain consistent results.

SUMMARY

(1) The doses of anti-serum required to neutralise various multiples of 1 d.c.l. of *Vipera russellii* venom have been determined and it has been observed that the amount of anti-serum required to neutralise 5 d.c.l. of venom is much greater than five times the amount of serum neutralising 1 d.c.l.

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(2) It has been observed that up to a level of 20 d.c.l. the relationship between the dose of venom and that of serum required to neutralise the venom is approximately linear.

(3) When a given volume of anti-serum against *Vipera russellii* venom is diluted with physiological saline, its potency is found to vary inversely with the dilution indicating thereby that neither the total number nor the activity of the antibody molecules or micelles is affected by dilution.

In conclusion, we wish to thank the authorities of the Bengal Immunity Co., Ltd. for supplying the antivenene serum.

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EFFECT OF REMOVAL OF LIPINS ON THE SOLUBILITY OF ANTIBODY PROTEINS AND ON THE REACTION BETWEEN ANTIGENS AND ANTIBODIES

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It has been shown by Wu (1) and Liu and Wu (2, 3) that the susceptibility of serum proteins to precipitation by salts like sulphates of sodium and magnesium is considerably increased, when the lipins present in the serum are removed. It is well known that the antibodies are usually precipitated with the pseudoglobulin fraction when an antiserum is concentrated. An investigation on the effect of removal of lipins on the solubility of antibody protein was undertaken with the object of exploring the possibility of concentrating antibodies from the immune sera. The results so far obtained with the antiserum against the venom of *Vipera russellii* are recorded in this paper.

EXPERIMENTAL

EFFECT OF SALTS ON THE PRECIPITATION OF ANTIBODY FROM LIPIN-FREE SERUM

A certain volume of an antiserum against the venom of *V. russellii* was taken and divided into two parts (A) and (B), the volumes of which were noted. (A) was kept as a control, while the lipins present in (B) were extracted by a method similar to that of Hewitt (4). The serum (B) was cooled below 0° and was vigorously shaken with 6 times its volume of an ice-cold mixture of ether and alcohol (3 : 7 by volume) for a short time. The mixture was then replaced in a cold chamber, maintained at −2° and left for 2 hours. The precipitated proteins were then washed several times with the ice-cold mixture of ether and alcohol and finally washed with pure ether. It was then suspended in pure ether below 0° and left in the cold chamber for 48 hours. It was afterwards filtered and dried in a vacuum desiccator. The dried powder thus obtained was dissolved in physiological saline and the protein content of the solution was adjusted at the level of the original serum (B).

One cc. of this extracted serum was made up to 10 cc. with physiological saline and the precipitation of proteins and antibody from each such 10 cc. solutions, by different quantities of sodium sulphate, has been determined. Exactly similar experiments were carried out with the serum (A) The results are recorded in Table I. It will be noticed that for the same concentration of sodium sulphate the percentage of protein and antibody precipitated from the extracted serum is considerably greater than that precipitated from the original serum. From the lipoid-free serum the entire amount of antibody is precipitated when the sodium sulphate concentration is 22.5 per cent while for the original serum 83.3 per cent of the antibody is precipitated under the same conditions. It will also be noticed that if the extracted serum is first adjusted at 17.1 per cent sodium sulphate concentration, filtered and the filtrate brought to 22.5 per cent sodium sulphate concentration, about 80 per cent of the antibody associated with only 30 per cent of the total protein is obtained. From the original serum under similar conditions 64 per cent of antibody associated with 40 per cent of protein is obtained. Therefore the purity of the antibody separated from the extracted serum is greater than that separated from the original serum.

TABLE I.

$pH = 6.9 - 7$

Sodium Sulphate	Wt. of N_2 in 100 cc. filtrate.		Nitrogen precipitated.		Antibody precipitated.	
	Original serum.	Extracted serum.	Original serum.	Extracted serum.	Original serum.	Extracted serum.
13.5%	1.0 g.	0.80 g.	7.4 %	23.77 %	Nil.	Nil.
14.4	0.960	0.720	11.11	30.76	,,	,,
15.3	0.920	0.680	14.45	34.65	,,	,,
16.2	0.880	0.640	18.51	38.46	,,	15.6 %
17.1	0.840	0.520	24.07	50.0	19.2 %	20.9
18.0	0.600	0.480	44.44	53.84	41.5	50.00
18.9	0.560 .	0.400	48.14	61.53	50.00	55.5
19.8	0.496	0.360	54.07	63.38	55.5	62.5
20.7	0.480	0.280	55.55	73.08	62.5	71.6
21.6	0.440	0.240	59.25	75.00	71.6	83.3
22.5	0.440	0.200	63.96	80.0	83.3	100.0
23.4	0.320	0.160	70.37	85.5	98.0	100.0

EFFECT OF REMOVAL OF LIPINS ON THE SOLUBILITY OF SERUM PROTEINS AT DIFFERENT pH

The effect of extraction of lipins on the solubility of serum proteins at various pH was also investigated. The extraction of lipins from the serum was carried out in exactly the same way as already described. The extracted as well as the original serum was diluted with physiological saline to 10 times their original volumes. Ten cc. portions from each of the two diluted sera

were placed in a series of test tubes and their pH were adjusted at the requisite level by the addition of an acid or an alkali. The proteins and the antibody precipitated were determined in each case. The results are recorded in Table II. It may be mentioned that protein content per cc. of the extracted serum was the same as that of the original serum. It will be noticed from the data recorded in Table II that at any given pH, the solubility of the protein in the extracted serum is much less than that in the case of the original serum. In both the cases, the maximum amount of protein is precipitated at pH 5.8. It will be noticed further that at any given pH, the percentage of antibody precipitated from the extracted serum does not differ appreciably from that obtained from the original serum.

TABLE II.

	Original serum (antivenene)			Extracted (antivenene)	
pH	Percentage of nitrogen pptd.	Percentage of antibody pptd.	pH	Percentage of nitrogen pptd.	Percentage of antibody pptd
5.0	6.8	nil.	4.9	20	nil.
5.4	9.0	nil.	5.6	22.3	nil.
5.8	10.9	nil.	5.8	26.2	nil.
6.1	7.4	9.1	6.0	23.1	10
6.5	5.4	9.1	6.4	16	9.0
7.2	2.2	nil.	7.2	7.6	nil.

EFFECT OF REMOVAL OF LIPINS ON THE REACTION BETWEEN *Vipera russellii* VENOM AND ITS ANTIVENENE

In a previous paper it has been reported by Ghosh and Kundu (5) that when the venom of *Vipera russellii* and its antivenene are mixed in different proportions in a series of test tubes the balanced mixture develops the maximum turbidity. Recently, Hartley (6), Horsfall and Goodner (7) and others noticed that removal of lipins affect the flocculation or the precipitin reactions between some antigens and their antibodies. Experiments were therefore undertaken to ascertain if the removal of lipins from the venom of *Vipera russellii* and its antivenene has any effect on the turbidity reactions as reported by Ghosh and Kundu (*loc. cit.*). The extraction of lipins from the venom and the antivenene was carried out in the same way as described in Table III. In all six samples of sera of which two were concentrated ones were tested. It will be noticed from the data that the removal of lipins has no effect on the development of turbidity. Furthermore, even with the extracted sera and venom the maximum turbidity was found to develop in the tubes containing the balanced mixtures. The *in vivo* tests were carried out using pigeons (weighing 300 gms.), as experimental animals and injections were given by the intravenous route.

TABLE III.

| Sample of antivenene. | Mode of treatment of | | Effect. | No. of M.L.D. neutralised per cc. of extracted serum. | |
	Antibody.	Antigen.		Turbidity test.	Vivo test. test.
x_1	Original	Original	Turbid	125	137
	Extracted	Extracted	,,	122	123
x_2	Original	Original	,,	248	270
	Extracted	Extracted	,,	250	260
x_3	Original	Original	,,	250	255
	Extracted	Extracted	,,	240	240
x_4	Original	Original	,,	128	135
	Extracted	Extracted	,,	120	125
x_5	Original	Original	,,	300	320
	Extracted	Extracted	,,	299	294
x_6	Original	Original	,,	465	480
	Extracted	Extracted	,,	450	470

SUMMARY

1. It has been found that the removal of lipins from the antiserum against the venom of *Vipera russellii* markedly affects the solubility of the antibody protein in the presence of sodium sulphate and that 80 per cent of the antibody can be concentrated in a protein fraction constituting 30 per cent of the total protein of the original serum.

2. The removal of lipins from the venom of *Vipera russellii* and its antiserum has no effect on the turbidity developed in solutions containing a balanced mixture of the antigen and the antibody.

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BIOCHEMICAL CHANGES IN EXPERIMENTAL SCURVY

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The ease with which ascorbic acid is oxidised and reduced points to its probable rôle as catalyst in biological oxidations and numerous experiments have been carried out with isolated tissues and enzymes to establish a connection between it and known dehydrogenase systems. Such experiments have not been successful in elucidating the physiological function of the vitamin. It appeared probable that a study of the biochemical changes in scorbutic animals might reveal some characteristic metabolic derangement associated with vitamin C deficiency.

This investigation is concerned with the urinary nitrogenous constituents, the glucose tolerance and the glycogen content of the liver of guinea-pigs in scurvy.

URINARY NITROGENOUS CONSTITUENTS

In studies on the nitrogen balance in experimental scurvy Shipp and Zilva (1) could not detect any disturbance in the absorption or retention of nitrogen due to the disease. The nitrogen balance became negative only after the intake of food diminished due to lack of appetite. Jarussova (2) found on the contrary that the nitrogen balance became negative in scurvy even when the animals were fed by force. Tomita (3) detected considerable increases in urinary nitrogen with the development of scurvy. In the present investigation no significant change was detected in the total nitrogen excretion in avitaminosis C. Further, contrary to the finding of Tomita, no increase in uric acid excretion could be detected. The ammonia excretion also remained unaffected. Contrary to the reported increase in excretion of creatinine in scurvy (4) an actual fall in the excretion of this constituent was noticed from about the twentieth day of deprivation of vitamin C. There was, however, a very noticeable increase in the excretion of creatine. This could not be attributed to insufficient food intake since in all cases the appetite of the animals remained unimpaired till the third week of the experiment while the increased excretion of creatine made itself evident much earlier.

BLOOD SUGAR AND SUGAR TOLERANCE

Sigal and King (5) reported a decrease in glucose tolerance in scurvy. Both the fasting blood sugar and the sugar content of the blood forty minutes after a glucose meal was found by them to be higher in scorbutic than in normal guinea-pigs. Palladin and Utewski (6) and Tomita (3) have similarly reported a higher fasting blood sugar level in scurvy. However, our observations go to confirm the earlier finding of Randoin and Michaux (7) who in numerous experiments found that scurvy does not affect the fasting blood sugar. But after ingestion of glucose the rate of fall of blood glucose from the maximum value was found to be noticeably slower in scorbutic than in normal animals. Even more apparent was a lowering in the rate of absorption of sugar after a glucose meal. Whereas in normal animals the highest blood sugar value was reached within an hour after the ingestion of glucose in scorbutic guinea-pigs it took two hours or more to reach the maximum value.

GLYCOGEN STORAGE IN THE LIVER

The partial failure in scurvy of the mechanism of glucose utilisation and the finding of Hermann (8) that injection of ascorbic acid into normal guinea-pigs resulted in increased deposition of glycogen in the liver led us to study glycogen storage in liver during scurvy. As rigorous control of food intake was necessary for this purpose the method of paired feeding was adopted.

All other conditions including food intake being identical, control animals were found to store up much more glycogen in the liver than scorbutic ones.

EXPERIMENTAL

Methods.—Guinea-pigs of the laboratory stock, 300-350 g. in weight, were used. The Sherman (9) basal diet was used with the difference that skimmed milk and cod liver oil were fed to the animals directly by pipette instead of admixing skimmed milk and butter fat with the rest of the food. The food trays were weighed each day and in cases where the food intake was found to fall off forced feeding was resorted to. Controls received 5 mg. and animals of the subscurvy group 0.25 mg. of ascorbic acid ('Redoxon' Roche) in addition to the basal diet. Urine collections were made in metabolism cages, toluene being used as preservative. Blood samples for analyses were drawn from punctures made in the margin of the ear, a little xylene being applied externally to stimulate blood flow when necessary.

Chemical Methods -

Glucose	...	Benedict's micro-colorimetric method.
Glycogen	...	Pfleuger's method modified by Good *et. al* (10)
Creatinine	...	Folin's modification of the Jaffe reaction.
Uric acid	...	Folin's colorimetric method.
Ammonia	...	Method of Van Slyke and Cullen.
Total and protein N		Parnas-Pregal micro-Kjeldahl method.

The probable errors were calculated according to the method adopted by Coward (11).

Urinary Nitrogenous Constituents.—Table I gives the variation in the excretion of creatinine and Table II the excretion of creatine in scurvy and latent scurvy. Table III gives the values of total and protein nitrogen, ammonia and uric acid in urine. There was no significant difference between normal and scorbutic guinea-pigs in the excretion of uric acid and ammonia. Some samples of scorbutic urine, however, contained traces of free protein. These were smoky in colour and contained traces of blood.

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TABLE I.

Excretion of Creatinine in the Urine

Days on diet.	Scorbutic.			Subscorbutic.			Normal.		
	No. of Animals.	Mg. of Creatinine.	Difference.	No. of Animals.	Mg. of Creatinine.	Difference.	No. of Animals.	Mg. of Creatinine.	Difference.
1	22	8.90					12	8.15	
3	13	8.08	0.82±0.50						
7	23	8.71	0.19±0.17				12	8.30	0.15±0.70
10	23	8.76	0.14±0.37	8	8.66		12	8.20	0.10±0.45
13	23	8.05	0.85±0.64	8	9.06	0.60±0.50			
17	23	7.50	1.40±0.31	8	8.90	0.24±0.39			
19							12	8.75	0.60±0.55
22	22	7.05	1.85±0.30				12	8.60	0.45±0.62
25	23	7.31	1.59±0.44				12	7.11	1.04±0.60
27				8	8.07	0.59±0.35			
40							12	9.40	1.25±0.74
42				8	7.90	0.76±0.97			
53				8	8.05	0.61±0.32			

TABLE II.

Excretion of Creatine in the Urine

Days on diet.	Scorbutic.			Subscorbutic.			Normal.		
	No. of Animals	Mg. of Creatine.	Difference.	No. of Animals.	Mg. of Creatine.	Difference.	No. of Animals.	Mg. of Creatine.	Difference.
1	22	0.10					12	Nil	
3	13	0.34	0.24±0.31				12	0.05	0.05±0.22
7	23	2.10	2.00±0.50						
10	23	2.41	2.31±0.62	8	0.11		12	0.10	0.10±0.20
13	23	4.45	4.35±0.60	8	0.31	0.21±0.51			
17	23	7.80	7.70±1.10	8	0.70	0.59±0.30			
19							12	0.45	0.45±0.40
22	23	8.84	8.74±1.88				12	0.10	0.10±0.38
25	23	10.64	10.54±1.35	8	0.68	0.57±0.44	12	0.35	0.35±0.32

TABLE III.

Total N, Protein N, Ammonia and Uric acid in Urine.

No. of animals in group.	Days on diet.	Total N (mg.)	Protein N (mg.)	Ammonia (mg)	Uric acid (mg.)
		Scorbutic			
23	1	102	Nil	6.80	2.01
23	3	180	Nil	4.13	2.15
23	7	140	Nil	6.00	2.20
20	12	155	Nil	5.70	1.85
23	15	172	Nil	7.15	2.05
23	22	135	32.0	7.25	2.45
3	30	220	47.3	7.10	2.78
		Subscorbutic			
8	10	176	Nil	5.10	1.88
8	13	271	Nil	4.90	2.10
8	27	182	Nil	5.25	1.85
8	42	171	Nil	5.75	1.76
		Normal			
12	1	180	Nil	3.80	2.60
12	10	163	Nil	4.22	2.12
12	19	188	Nil	5.15	2.80
12	22	170	Nil	4.70	2.54
12	40	197	Nil	4.55	2.85

Sugar Tolerance.—The animals were fasted for four hours preceding the test. A weighed amount of glucose (0.3 g. per 100 g. body weight) was fed orally. Blood samples were withdrawn every half an hour and the glucose content determined. The results are given in Table IV and graphically in Figure 1.

FIG. 1

TIME IN HOURS
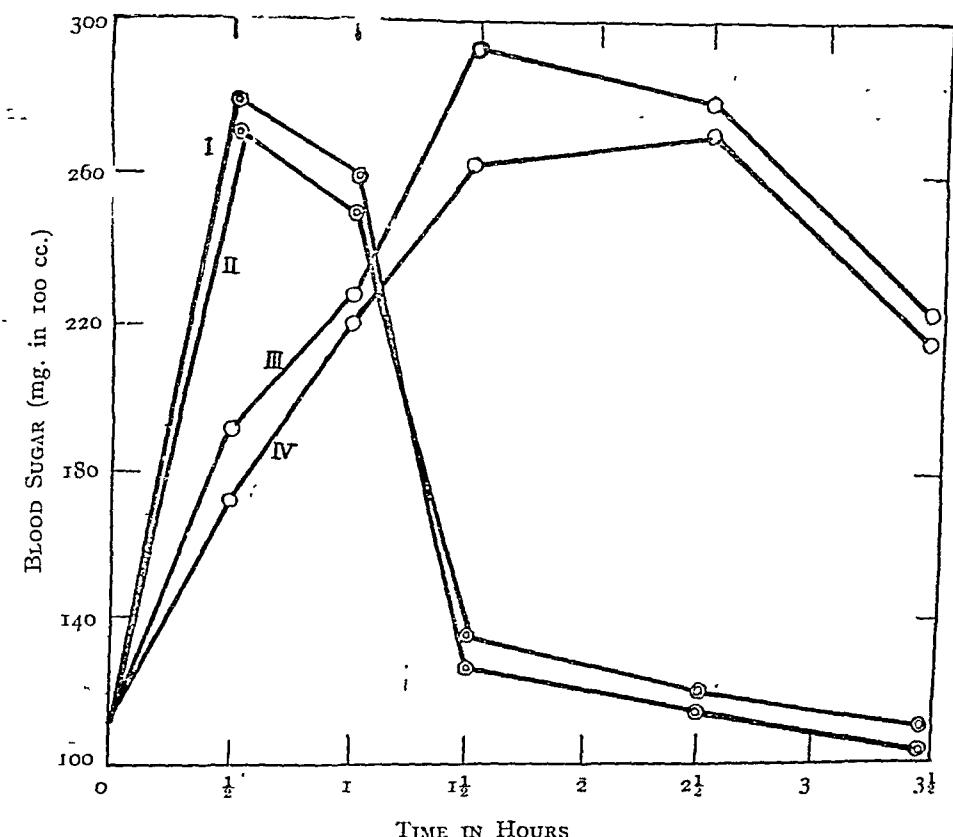
Glucose Tolerance in Scurvy

I.   10 days on Scorbutic diet.
II. 18 ,,   ,,   ,,   ,,
III. 24 ,,   ,,   ,,   ,,
IV. 26 .,,   ,,   ,,   .,

TABLE IV.

(Blood sugar in mg. of glucose in 100 ml. blood)

No of animals tested.	No. of days on diet.	Blood-sugar after administration of glucose					
		Before	$\frac{1}{2}$ Hr.	1 Hr.	$1\frac{1}{2}$ Hr.	$2\frac{1}{2}$ Hr.	$3\frac{1}{2}$ Hr.
		Scorbutic animals.					
6	10	115±2.2	272±0.9	251±3.7	135±1.5	120±1.5	110±2.1
12	18	112±1.0	280±1.8	261±2.2	126±1.3	115±6.5	107±1.8
12	24	115±3.8	172±2.6	223±1.9	268±2.3	276±5.2	201±3.7
12	26	116±4 2	194±5.2	228±6.1	302±1.4	281±7.1	211±3.7
		Subscorbutic animals.					
8	30	115±4.1	292±3.0	247±5.2	220±8.5	180±5.0	130±2.9
8	40	118±2.5	282±2.2	250±3.9	226±2.0	200±9.3	177±4.9
8	52	114±1.8	290±6.7	272±3.0	230±2.7	175±7.3	137±4.1
		Normal Controls.					
10	23	117±6.2	280±4.4	171±6.7	150±3.1	130±4.9	112±2.4
7	50	112±5.7	285±3.8	186±7.0	153±1.7	122±5.0	105±6.1

Glycogen Storage in the Liver.—For a group of four scorbutic guinea-pigs exact controls were maintained, the paired feeding method being used to eliminate any error due to differences in food consumption. On the 18th day of the experiment the animals were killed, the liver removed at once and the glycogen content determined by the method of Good *et. al* (10). To obliterate any differences introduced by the presence of different quantities of food in the gut a starvation period of 12 hours preceded the killing. The results (Table V) show that there is a marked fall in the glycogen storage of the liver in scurvy. Under the conditions of the experiment this cannot be due to differences in food intake. Further experiments on the synthesis of glycogen in the scorbutic liver are in progress.

TABLE V.

Glycogen Content of Liver in Scurvy.

Description of animal.	Body-weight.	Wt. of liver.	Total Glycogen	Glycogen per g.
Scorbutic	260 g.	9.2 g.	22.0 mg.	2.4 mg.
Control	320	13.1	157.3	12.1
Scorbutic	280	11.8	73.1	6.2
Control	330	12.3	242.8	19.7
Scorbutic	300	12.0	60.2	5.0
Control	335	13.8	281.0	20.4
Scorbutic	240	9.8	33.7	3.44
Control	300	11.2	128.6	11.48

SUMMARY

A biochemical study of the effect of scurvy on the metabolism of guinea-pigs has been carried out with the following results:

1. There is no variation from the normal in urinary excretion of total nitrogen, ammonia and uric acid.

2. The output of creatinine decreases while there is a large increase in the excretion of creatine.

3. The glucose tolerance is lowered.

4. There is a marked decrease in the glycogen content of the liver.

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A COMPARATIVE STUDY OF THE CHEMICAL AND BIOLOGICAL METHODS OF ESTIMATION OF VITAMIN C OF CABBAGE JUICE

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(Received for publication May 7, 1941)

A method of estimating "total" ascorbic acid in food-stuffs involving the heating of the aqueous suspension in hydrogen sulphide atmosphere and treatment with "ascorbic acid oxidase" has been described before (1). In a number of substances, the values obtained by this method have been found to be higher than those obtained by the trichloroacetic acid extraction method of Tillmans and Harris. This is because in our view this method takes into account, apart from ascorbic acid, ascorbigen (or combined ascorbic acid) and dehydroascorbic acid, if present (1). Although the chemical method thus appears to support the idea that a small part of ascorbic acid in certain food-stuffs is present as ascorbigen, it was desirable to test this view by biological methods. Unfortuntely the biological method does not usually permit detection of differences of activity which are likely to occur. In our preliminary experiments with guinea-pigs by the growth method, the variations were indeed found to be too great and desultory to permit any conclusion to be drawn. Experiments were therefore undertaken with a fairly large number of guineapigs by the tooth method which has been found to give somewhat more uniform results, and a single food-stuff, the cabbage, was selected for systematic investigation, as it has already been reported to be a fairly good source of ascorbigen.

The present investigation was planned to find which of the different chemical methods of estimating ascorbic acid, namely, (1) the simple trichloroacetic acid method, (2) the method based on reduction with hydrogen sulphide in the cold to reduce any dehydroascorbic acid, if present, and (3) the method developed by us based on treatment with hydrogen sulphide in hot solution in order to split up ascorbigen and reduce dehydro-ascorbic acid, if present, would give values of ascorbic acid nearest to those determined biologically. For this purpose fresh cabbage juice was taken every day and its ascorbic acid content was determined chemically by the three methods mentioned above. Different volumes of this extract containing the same sub-optimal dose of ascorbic acid (0.25 mg.) were fed to three groups of guineapigs and the same amount of pure ascorbic acid was fed to a separate

group of guineapigs. The histological tooth results obtained with the last group of guineapigs were compared statistically with those obtained by the other three groups. Thus the volume of cabbage juice which gave results most approximate to those obtained by feeding 0.25 mg. of pure ascorbic acid was determined. It was thus possible to find which of the chemical methods gave the most correct value of ascorbic acid of cabbage which was nutritionally available.

EXPERIMENTAL

Male guineapigs weighing between 150 and 170 g. were given a mixed diet of green grass and germinated gram *ad libitum* for about 21 days. When the body weight rose between 200 and 250 g., they were placed on a scorbutic diet consisting of oats 65 parts, gram 20 parts, casein 12 parts, common salt 1 part, calcium carbonate 3 parts, codliver oil 2 cc. and tap water *ad lib.*

Sixty animals were divided into 4 groups, 15 being in each group. In each cage which was sufficiently large 5 animals were kept. The experiments were performed in the month of April 1940, when the room temperature varied from 81°F to 103°F. Within a period of 15 to 20 days the animals began to lose weight evenly and showed early symptoms of scurvy, and when they had lost weight to the extent of 10 to 20 g. from their maximum weight during 3—4 days, their diet was supplemented with cabbage juice. *Any animals whose weight curves were found to be irregular were discarded.*

The ascorbic acid content of cabbage juice was estimated by the following different methods:

Method I. Estimation of ascorbic acid of cabbage juice by simple trichloroacetic acid method.—A portion of fresh cabbage was cut into small pieces by means of a knife which had previously been moistened with 20% trichloroacetic acid in order to minimise action by the ascorbic acid oxidase of cabbage. The small pieces were then placed in a glass mortar and immediately moistened with 3 or 4 drops of the same acid. It was then finely ground by triturating with washed sea-sand. The juice was then squeezed out through a clean cotton cloth for the estimation of ascorbic acid. This may not correspond to the ascorbic acid value of the fresh cabbage, which should normally be ground under 20% trichloroacetic acid, but it is the juice of cabbage estimated under different comparable conditions, which we were concerned with in the present investigation. To 5 cc. of the juice 2.5 cc. of 20% trichloroacetic acid were added ; the mixture was diluted to 100 cc. and titrated against 2 : 6-dichlorophenol-indophenol (2). Ten cc. of the solution were brought to *p*H 5.6 by the addition of dilute sodium hydroxide solution. Two cc. of acetate buffer of the same *p*H and 2 cc. of ascorbic acid oxidase solution were then added to it. This was incubated at 40° for half an hour, made up to a definite volume and titrated against the dye. The difference between the first and second titration values gave the amount of ascorbic acid in the juice.

Method II. *Estimation of ascorbic acid of cabbage juice by cold hydrogen sulphide method.*—The fresh cabbage was minced by means of a mincer and then the juice was pressed out through clean cotton cloth. A portion of the juice was kept in an airtight conical flask through which· air had been displaced by a current of carbon-dioxide in order to prevent aerial oxidation. This sample was kept in a refrigerator for about 2 to 3 hours for feeding the animals.

Five cc. of the filtered juice were taken in a 250 cc. conical flask. It was diluted to 50 cc. and hydrogen sulphide was passed through it for about 20 mins. which was then chased out by a current of carbon-dioxide .or coal gas (previously bubbled through chromic acid solution). These gases were found in separate experiments not to affect the ascorbic acid value. When the last traces of hydrogen sulphide had been removed, 2.5 cc. of 20% trichloroacetic acid were added and the solution was diluted to 100 cc. It was then titrated against the dye by the use of oxidase as described above. This method gave the amount of free ascorbic acid and dehydroascorbic acid.

Method III. *Estimation of ascorbic acid of cabbage juice by hot hydrogen sulphide method.*—Five cc. of the cabbage juice were diluted to 50 cc. in a 250 cc. conical flask and were treated with hydrogen sulphide successively for 5 minutes in the cold, 10 minutes on a boiling water-bath and again for 5 minutes in the cold. After the removal of hydrogen sulphide, the solution was titrated as before. This method gave the total amount of free ascorbic acid, dehydroascorbic acid and ascorbigen present.

Method IV. *Biological*: (*a*) *Administration of the juice.*—The guinea-pigs were weighed every day at a definite time. They were fed every day by means of a 1 cc. graduated pipette at a definite time. The doses given to the different groups of animals were as follows:

Group 1   ...   X cc. of cabbage juice equivalent to 0.25 mg. of ascorbic acid daily as estimated by simple trichloroacetic acid method.

Group 2   ...   Y cc. of cabbage juice equivalent to 0.25 mg. of ascorbic acid daily as estimated by cold H_2S-method.

Group 3   ...   Z cc. of cabbage juice equivalent to 0.25 mg. of ascorbic acid daily as estimated by hot H_2S-method.

Group 4   ...   0.25 mg. of pure ascorbic acid.

(*b*) *Determination of histological changes in teeth.*—After 12 days the animals were killed by giving a blow on the head. The incisors of the lower jaw of guineapigs were taken out and freed as completely as possible from the adhering muscle and skin. Teeth were then fixed in a normal formal-saline solution of the composition 0.9 g. sodium chloride, 10 cc. of 40% formalin and 90 cc. water. Twenty-four hours' immersion was sufficient for fixing.

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The teeth were decalcified by a solution of the composition: 5 c strong nitric acid, 50 cc. saturated potash alum solution and 50 cc. distille water. About 100 cc. of decalcifier were used for a pair of teeth. Th decalcifier was removed after 24 hours. The process was repeated with fresh sample of decalcifier till completion of decalcification, which wa ascertained by inserting a fine needle. It would give a gritty feel if an calcified matter remains. The time needed for complete decalcification varie from 3 to 15 days.

The tissue was then subjected to dehydration by immersing successivel in 50%, 70% and 95% alcohol for one hour in each case and final dehydra tion was effected by means of absolute alcohol. The teeth were then cleare by immersion in cedar wood oil, which serves also to preserve the teeth fo some time before sections are cut.

The part required for examination was the root of the incisor in th region of the bend of the jaw just in front of the first molar (3). The part not needed were then cut away by means of a sharp blade. The tissue wa then embedded in a molten paraffin bath at 60° for about 6 hours under vacuum in order to expel the air from the tissue so that paraffin might penetrate inside the tissue. After the completion of the process the tooth was placed in a small paper box containing molten paraffin of m.p. 56°—58°, so that the cut surface was parallel to the base of the box. The box was then immediately put in a bath of ice.

The paraffin block thus prepared was then mounted on the microtome and the sections were adjusted to 15μ thick and the sections were taken from the desired point by trial. Next day the paraffin was washed by means of xylene and then stained with hæmatoxylin and eosin. The sections were dehydrated in alcohol and cleared in xylene and cover-slips were attached with Canada balsam as usual. The slides were then examined under microscope and degree of protection was determined from the histological changes produced in the teeth of experimental guineapigs receiving different doses of vitamin C. The results were compared with the arbitrary protection scale of Key and Elphick (4) depending on the disorganisation of odontoblast layer, the condition of inner dentine and predentine and the position of Tomes' canals. The cross-section of a normal tooth consists of pulp in the middle surrounded by a layer of long parallel odontoblasts, a narrow un-calcified predentine and a wider dentine. Fine Tomes' canals run from odontoblasts through predentine and dentine. In a scorbutic tooth the odontoblast layer is disorganised, the predentine calcified, an irregular layer of bone known as inner dentine is formed and the Tomes' cannals are fewer and are only found radiating from predentine outwards.

The following table shows the degree of protection conferred on the different experimental animals by the cabbage juice.

TABLE I.

No. of animals.	Group 1 Animals receiving 0.25 mg. of ascorbic acid equivalent cabbage juice daily as estimated by simple trichloroacetic acid method. (Method I).	Group 2 Animals receiving 0.25 mg. of ascorbic acid equivalent cabbage juice daily as estimated by cold—H_2S method. (Method II).	Group 3 Animals receiving 0.25 mg. of ascorbic acid equivalent cabbage juice daily as estimated by hot—H_2S method. (Method III).	Group 4 Animals receiving 0.25 mg. of pure ascorbic acid daily. (Standard Method IV).
1.	1.50	1.50	1.50	1.00
2.	2.50	1.00	1.50	1.00
3.	0.50	1.50	1.00	0.75
4.	2.50	1.50	1.00	0.50
5.	0.75	1.50	0.50	1.50
6.	2.00	1.00	2.00	1.50
7.	2.50	1.00	1.00	1.00
8.	3.50	1.50	0.50	1.50
9.	1.50	1.00	1.50	0.50
10.	0.50	1.50	1.50	1.00
11.	1.50	1.00	0.50	1.00
12.	0.50	1.50	0.50	1.00
13.	1.00	1.50	0.50	1.00
14.	—	—	1.00	—
15.	—	—	1.00	—
Mean	1.596	1.3087	1.033	1.02

These figures were kindly subjected to a statistical analysis by Mr. K. C. Basak and the statistical note is given below:

The means of the degrees of protection as estimated by the four different methods are given in Table II.

TABLE II.

Mean degree of protection

Method	Mean degree of protection
I	1.60
II	1.31
III	1.03
IV	1.02

The means differ among themselves. The maximum value is 57 per cent more than the minimum value, the latter being the standard estimate. The existence of a real difference in response to the four methods can, however, be established only in the absence of similarity between the variation of the means and the variation of the response in the individual animals. This may be investigated by an analysis of variance which is shown in Table III.

TABLE III.

Analysis of variance of the degree of protection.

Variance due to	Degrees of freedom.	Sum of squares.	Mean square	Ratio
Method	3	2.9790	0.9930	3.05
Individual	50	16.2525	0.3251	
Total	53	19.2315	0.3629	

The variance due to the difference in the methods is more than three times the variance which may be ascribed to the behaviour of the individual animals. This ratio is significant at the 5 per cent level. Hence the differential effect of the four methods is real.

We shall now investigate the difference between Method IV, which is the standard, and each one of the other three methods. The analysis is shown in Table IV.

TABLE IV.

Analysis of difference of means by pairs.

Difference between methods.	Difference of means.	Standard error of difference.	t
I and IV	0.58	0.2236	2.59
II and IV	0.29	0.2336	1.30
III and IV	0.01	0.2160	0.05

The standard errors are based on 50 degrees of freedom and the corresponding expected values of t are as follows:

$$t > 1.96 \quad \text{at} \quad 5\% \quad \text{level}$$
$$t < 2.46 \quad \text{at} \quad 2\% \quad \text{level}$$

Hence only the difference between Mthods I and IV is significant.

It may be noted that in the last two cases of Table IV the standard errors are fairly high as compared with the differences of the means. This is due to the high variability of the animals used in Method I, which may be clearly seen from Table V.

TABLE V.

Variance due to individual variations.

Method.	Degrees of freedom.	Variance.
I	12	0.9119
II	12	0.0641
III	14	0.2611
IV	12	0.1090

The variance in Method I differs significantly from the variances in all other cases. The variance in Method II also differs significantly from that in Method III. It would be, therefore, reasonable to compare Methods II and IV on the basis of the standard error of difference calculated from the individual variations of the animals used only in these two methods. The corresponding analysis is shown below:

Difference between	Difference of means.	Standard error of difference.	t
Methods II and IV	0.29	0.1153	2.52

The expected value of t for the available 24 degrees of freedom is 2.49 at the 2% level. Hence the difference is significant. This conclusion, however, is to be regarded as a tentative one and should be verified by results obtained from a repetition of the experiment according to Methods II and IV.

A similar analysis of the difference between Methods III and IV gives an observed value of t which is less than unity. Hence the difference is not significant.

To sum up, Method I differs significantly from the standard method. A similar difference probably exists between Method II and the standard method, but the evidence is not conclusive. There is no real difference between Method III and the standard method.

DISCUSSION

Tests carried out with guineapigs are known to show wide individual biological variations. As the purpose of this investigation was to obtain biological evidence regarding the accuracy of different methods for the estimation of "total" ascorbic acid, comprising free ascorbic acid, dehydro-ascorbic acid and ascorbigen, the present experiments with guineapigs were carried out under carefully controlled conditions and statistically analysed.

From the statistical note it appears that the results obtained with animals of group III by feeding daily cabbage juice estimated to contain 0.25 mg. ascorbic acid by Method III and the results obtained with animals of group IV by feeding 0.25 mg. pure ascorbic acid daily (Method IV) agree very closely. On the other hand results obtained with animals of group II which were receiving cabbage juice equivalent to 0.25 mg. ascorbic acid daily as estimated by Method II seemed to differ appreciably from those obtained by feeding 0.25 mg. pure ascorbic acid. What is particularly noteworthy is that the results obtained by feeding animals of group I with doses of cabbage juice estimated to contain 0.25 mg. ascorbic acid by simple trichloroacetic acid method (Method I) are significantly different from those obtained by feeding pure ascorbic acid. The trichloroacetic acid method (Method I)

would not take into account dehydroascorbic acid and ascorbigen. The cold-H₂S method (Method II) would take into account the former but not the latter, while the hot-H₂S method (Method III) would take into account of free, dehydro- and combined ascorbic acid. The results obtained with guineapigs present in this paper would appear to lend support to the view that Method III as described by us (I) involving the use of hydrogen sulphide in hot condition and the use of ascorbic acid oxidase, would give approximately the most correct value of "total" ascorbic acid. Nutritionally this is important, as all the three—ascorbic acid, dehydroascorbic acid and ascorbigen—are physiologically available.

Our preliminary work with guineapigs by the growth method gave very much wider individual variations than the tooth method, which probably is more specific.

SUMMARY

Different volumes of cabbage juice estimated to contain 0.25 mg. ascorbic acid by three different chemical methods *viz.* (1) simple trichloroacetic acid method, (2) cold-H₂S method and (3) hot-H₂S method were fed daily to three groups of guineapigs on a scorbutic diet. A fourth group was given 0.25 mg. pure ascorbic acid per animal per day. The tooth method showed that the results obtained with the fourth group agreed most closely with those obtained with the third group of animals which were receiving cabbage juice estimated to contain 0.25 mg. ascorbic acid by the hot-H₂S method. The results obtained by the trichloroacetic acid method differed significantly from those obtained by feeding equivalent quantities of pure ascorbic acid. The method described before (I) involving the use of hot H₂S and of ascorbic acid oxidase is thus considered to give approximately the most correct value of total ascorbic acid comprising free ascorbic acid, dehydroascorbic acid and ascorbigen, all of which are nutritionally available.

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AVAILABLE IRON IN FISH—PART II.
THE NATURE OF IRON IN FISH TISSUE

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In a previous publication (1) we have investigated the action of proteolytic enzymes (pepsin and trypsin) on fish tissue in order to throw light on the question regarding the nutritional availability of the iron content of the tissue. From the results it appears that some of the iron of fish tissue is present as an organic iron-complex, which as such, does not react with α α' dipyridyl, but which is readily hydrolysed by pepsin and trypsin and should therefore be nutritionally available. After digestion, the iron liberated from the iron-protein complex can be estimated by the dipyridyl method of Hill. On this basis a method of estimation of the total available iron has been developed which involves peptic digestion followed by reduction with sodium hydrosulphite and treatment with dipyridyl. It was also found that in this method the substitution of peptic digestion by extraction with 10 per cent acetic acid served an equally good purpose. The value of ionisable iron thus obtained is higher than that obtained by the straightforward Hill's method.

It is not yet clear how far the ingested ionisable iron as determined by Hill's method is retained and is available for hæmoglobin formation. Some workers have observed that iron in certain foodstuffs is more potent in hæmoglobin formation than the pure iron salts, when incorporated in the diet at the same iron level (Hahn and Whipple, 2). It appears, therefore, that the elucidation of the nature of the non-hæmin iron which is present in foodstuffs is likely to lead to a better understanding of this question of availability.

Among different foodstuffs considerable work has been done regarding the iron of the egg. Nearly all the iron of hen's egg is located in the yolk and many believe that it exists in the form of an organic complex with protein-like properties. Miescher (3) observed that after prolonged peptic digestion of egg-yolk an insoluble substance separated out, which was found by Bunge (4) to contain iron in organic combination and was named "hæmatogen" by him. Hugounenq and Morel (5) regarded "hæmatogen"

as the prosthetic group in the vitellin molecule and as being the precursor of the hæmoglobin formed by the chick during incubation of the egg. They found that decomposition by acids leads to a black pigment containing 2.6% of iron.

Hill (6), by applying his own test to egg-yolk, concluded that all its iron is present as inorganic ferric iron and no organic complex exists. McFarlane (7), however, found that lecitho-vitellin prepared from egg-yolk contains a fair percentage of iron which appears to be present in a very stable combination. He, therefore, concluded that part at least of the iron of the egg is present as an organic complex. Commenting on Hill's results, McFarlane says that there is no evidence that $\alpha \alpha'$-dipyridyl reacts only with inorganic ferrous compounds and does not combine with ferrous organic compounds other than reduced hæmatin and, therefore, Hill's deduction about inorganic iron is not justified. The ferrous salt of dipyridyl, according to Blaw (8) is $[Fe(C_{10}H_8N_2)_3]X_2$, where X may represent a univalent inorganic or organic anion. Tompsett (9) found that hen's egg contained ferric iron which was probably in combination with phosphatides.

Working on several plant and animal tissues Lintzel (10) and Heubner (11) have stated that ferric (but not ferrous) iron forms stable complexes with proteins and consider that iron must be reduced to the ferrous condition before absorption from the intestine. McFarlane (12) is of opinion that autoproteolytic changes in liver and spleen at pH 4.5 also include the decomposition of organic iron-containing compounds, presumably iron proteinates. This decomposition is accelerated by hydrogen sulphide and inhibited by copper. During prolonged autolysis there takes place a recombination of iron with organic substances presumably proteose in nature, the magnitude of which is dependent upon the amount of iron liberated by proteolysis.

The present communication seeks to throw light on this still obscure question of the nature of non-hæmin iron in tissues and as our earlier work concerned mainly fish tissue (1) we have sought to investigate the nature of this iron in fish tissue with simultaneous experiments on duck's egg for comparison.

EXPERIMENTAL

Experiments of Tompsett (9) with hen's egg indicate that the amount of iron liberated by sodium hydrosulphite, thiolacetic acid or sodium pyrophosphate is the same. In our experiments the iron content of duck's egg was studied using thiolacetic acid and ammonia as the colour producing agent.

The yolk of one egg was diluted to 100 cc. with distilled water and well mixed. Three 15 cc.-portions of the sample were taken in test tubes and they were respectively treated with (i) 0.2 cc. of thiolacetic acid, (ii) 0.5 g. of sodium hydrosulphite dissolved in 5 cc. water and (iii) 5cc. of 4% sodium pyrophosphate solution. Each solution was diluted to 30 cc. with water. Another 15 cc. portion of the yolk sample was diluted to 30 cc. and kept as a control.

After standing for 5 minutes, 15 cc. of 20% trichloroacetic acid were added to each of the four solutions, the mixture well shaken and centrifuged. The residue was washed and centrifuged three times with 5 cc. portions of 5% trichloroacetic acid. To the filtrate and washings 6 drops of thiolacetic acid were added and the solution was made alkaline by adding 4 cc. of concentrated ammonia, when the colour was produced. The precipitate formed was filtered off and the colour of the filtrate was compared in a colorimeter with a standard solution of iron similarly treated.

The same experiments were repeated with 10 g. portions of minced fish tissue thoroughly mixed with 10 cc. of distilled water in each case. In each set of experiments, the same sample of fish was used.

The total iron content of 15 cc. of the diluted egg-yolk prepared as mentioned before and of 10 g. of the fish sample were determined after ashing accordingly to the method previously described. The results obtained with fish and egg are given in Table I.

The above results indicate that in the case of duck's egg-yolk, the amount of iron liberated by thiolacetic acid, sodium hydrosulphite or sodium pyrophosphate is practically the same which confirms the findings of Tompsett with hen's egg. As the control gives no test for iron, it indicates the absence of simple inorganic or organic ionisable form of iron which, if present, would have been presumably extractable with trichloroacetic acid, and would have given the colour when subsequently treated with thiolacetic acid and ammonia. Simple inorganic and organic iron salts such as ferric sulphate, ferric chloride, ferric nitrate, and ferric citrate have been tested with trichloroacetic acid and found to be fairly soluble. It appears, therefore, that simple trichloroacetic acid extraction does not remove the iron of the egg, which can, however, be liberated by previous treatment with the reagents mentioned above. This liberation is almost quantitative, as the values agree with the iron values obtained by the ashing of the egg-yolk.

But in the case of fish, the iron value obtained by reduction with sodium hydrosulphite is less than that obtained by thiolacetic acid. This is probably because reduction with sodium hydrosulphite requires a higher acidity. We have observed before (1) that if extraction is made with 8% acetic acid instead of with buffer at pH 5.5, the iron values obtained by reduction with sodium hydrosulphite agree fairly with those obtained by reduction with thiolacetic acid. When the fish sample is treated with sodium pyrophosphate only about 30 to 40 % of the iron estimated by the above methods can be accounted for. The control, namely the trichloroacetic acid extract, gives the test for iron but the amount is very small. Moreover, the amount of iron determined after ashing is always greater than that obtained by any. of the above methods. This probably indicates the amount of hæmatin iron associated always with the tissue.

II

TABLE I.

Iron in mg. per WHOLE EGG* and 100 g. of raw-fish.

Sample No.	Egg					Fish					
	Ionisable Iron					Bengali name.	Zoological name.	Ionisable Iron			
	Thiolacetic acid method.	Sodium hydro-sulphite method.	Sodium pyrophos-phate method.	Control.	Total iron (after ashing).			Thiolacetic acid method.	Sodium hydro-sulphite method.	Sodium pyrophos-phate method.	Control.	Total iron (after ashing).
1.	1.30	1.28	1.30	nil.	1.31	Rohit	Labeo rohita	0.56	0.35	0.28	0.12	0.71
2.	1.23	1.21	1.21	nil.	1.21	Mrigal	Cirrhina mrigala	0.88	0.59	0.32	0.09	0.92
3.	1.35	1.35	1.37	nil.	1.36	Boal	Wallago attu	1.25	0.85	0.30	0.02	1.5
4.	1.20	1.18	1.21	nil.	1.19	Catla	Catla catla	0.78	0.61	0.23	negligible.	0.98
5.	1.27	1.23	1.24	nil.	1.23	Ilish	Clupea ilisha	0.99	0.72	0.33	0.06	1.30

*(.10—.15g.)

Added iron forms complexes with fish and egg.—In order to see whether any added iron in the form of soluble salts either in ferric or ferrous condition forms complexes with substances in the egg-yolk and with fish tissue the following experiments were adopted:—

(1) Ferric chloride corresponding to 0.1 mg. of iron was added to 15 cc. of egg-yolk and extracted with trichloroacetic acid. The extract gave negative test for iron. The amount of added iron could be quantitatively recovered by the addition of thiolacetic acid, sodium hydrosulphite or pyrophosphate to the residue.

(2) Ferrous ammonium sulphate corresponding to 0.1 mg. of iron when added to egg-yolk behaved similarly, which is probably because ferrous iron hardly had any time to remain as such but was rapidly oxidised to the ferric form, which probably combined with protein and thereby became non-extractable with trichloroacetic acid, in which ferrous ammonium sulphate as such is easily soluble.

(3) Ferrous ammonium sulphate equivalent to 0.1 g. of iron was added to egg-yolk in an atmosphere of nitrogen. By subsequent extraction with trichloroacetic acid about 70% of the added iron could be obtained in the trichloroacetic acid extract. This indicates that ferrous salts cannot form complexes with egg-yolk.

All the above investigations were carried out also with fish tissue which behaved in a like manner.

Enzymatic hydrolysis of fish and egg and estimation of the iron in the hydrolysate.—To investigate whether the iron liberated from fish and egg on hydrolysis by pepsin (1) is in soluble form, four 15 cc. portions of egg-yolk were taken in different flasks and adjusted to pH 2.0 with hydrochloric acid. Ten cc. of 0.4% pepsin solution were added to each flask and the mixture incubated for 4 hours at 37°. One of the flasks was kept as control without the addition of the enzyme. After the incubation period was over the protein was precipitated with trichloroacetic acid and centifuged off. The extract in each case gave negative test for iron, showing that the iron liberated by peptic digestion is not soluble in trichloroacetic acid. The same results were obtained also in the case of fish tissue, the only difference being that the control in the latter case gave the test for iron which was apparently extracted by the hydrochloric acid, the amount being small in comparison with the total iron.

Addition of iron to egg-yolk and subsequent hydrolysis by pepsin did not give any test for iron in the hydrolysate, whereas in the case of fish the added iron could be almost quantitatively recovered in the extract obtained after peptic hydrolysis and trichloroacetic acid extraction. The control without pepsin gave a trichloroacetic acid extract which did not contain the added iron, which was therefore apparently fixed by the fish tissue. This indicates a very important difference between the iron complexes of egg-yolk

and those of fish tissue. The results obtained with fish tissue are given in Table II.

TABLE II.

Iron in mg. per 100 g. of fish tissue.

Bengali name.	Zoological name.	Iron in the original fish tissue.	Iron added.	Iron in the hydro-lysate.	Iron in control.	Iron recovered.
Rohit	*Labeo rohita*	0.82	0.20 mg.	0.43 mg.	0.24 mg.	0.19 mg.
Mrigal	*Cirrhina mrigala*	0.73	0.30	0.38	0.37	0.66
Boal	*Wallago attu*	0.63	0.30	0.79	0.48	0.31
Catla	*Catla catla*	0.71	0.40	0.61	0.24	0.37
Ilish	*Clupea ilisha*	0.83	0.40	0.68	0.32	0.36
Vetki	*Latas calcifer*	0.69	0.50	0.73	0.25	0.48

Enzymic hydrolysis and increment of acidity do not increase the iron value of egg as in fish and other animals tissues.—In the previous paper (1) we have found that hydrolysis of fish tissue and goat's muscle with pepsin, prior to reduction by sodium hydrosulphite and subsequent extraction with acetate buffer at pH 5.5 or reduction and extraction with 8 to 10% acetic acid instead of with the buffer, increased the values for ionisable iron to a great extent as determined by the dipyridyl method. But when the above experiments were carried out with egg-yolk, the iron value remained the same as that obtained by Hill's original method. In estimating the ionisable iron content of eggs therefore, the modification of Hill's method is not necessary.

Effect of ascorbic acid on the liberation of iron from egg and fish.—When ascorbic acid is added to egg-yolk or fish tissue instead of sodium-hydrosulphite or thiolacetic acid, some portion of the complex iron is liberated due to the reducing action of the acid. These results are shown in Table III. If it is a fact that before absorption by the system, the iron present in the foodstuff has to be reduced to the ferrous condition, then it seems probable that there may be some biochemical relation between vitamin C content of the body and iron utilisation. This aspect of the question is being studied further.

TABLE III.

Iron in mg. per 100 g. of raw fish and per whole egg.

	Iron in egg.		Iron in fish.			
Sample No.	Iron with vitamin C.	Iron by modified Hill's method.	Bengali name.	Zoological name.	Iron with vitamin C.	Iron by modified Hill's method.
1.	0.80 mg.	1.53 mg.	Rohit	*Labeo rohita*	0.63 mg.	0.93 mg.
2.	0.73	1.32	Boal	*Wallago attu*	0.82	1.21
3.	0.95	1.61	Vetki	*Latas calcifer*	0.50	0.86
4.	0.71	1.28	Mrigal	*Cirrhina mrigala*	0.56	0.92

Iron content of the residue left after prolonged enzymic hydrolysis of the fish meal.—It has already been stated that after enzymic hydrolysis of fish meal for 4 hours, the filtered hydrolysate does not give any test for iron except that which is liberated by hydrochloric acid at pH 2.0. Even if the period of hydrolysis is increased up to 24 hours, this iron value is not increased. The residue, left behind, gives the value for about 30 to 45% of the total ionisable iron as estimated by thiolacetic acid or by Hill's method as modified by us by previous treatment with 8% acetic acid (1). The results are indicated in Table IV.

TABLE IV.

Iron in mg. per 100 g. of fish tissue.

Sample No.	Iron in filtered hydrolysate after enzymic hydrolysis of the tissue for 24 hours.	Iron in the control with hydrochloric acid.	Iron in residue by Hill's modified method.	Iron in residue by thiolacetic acid method.	Iron in the original fish tissue by Hill's modified method.	Iron in the original fish tissue by thiol-acetic acid method.
1.	0.42 mg.	0.43 mg.	0.34 mg.	0.35 mg.	0.79 mg.	0.76 mg.
2.	0.65	0.63	0.32	0.31	0.96	0.98
3.	0.40	0.41	0.21	0.21	0.63	0.64
4.	0.47	0.47	0.23	0.21	0.70	0.70
5.	0.68	0.65	0.40	0.41	1.10	1.15
6.	0.72	0.73	0.48	0.46	1.25	1.20

From the above, it is clear that 30 to 40% of the so-called available iron is present in a very stable combination which resists the action of pepsin and hydrochloric acid at pH 2.0. It will be of interest to investigate the nature of combination of this organic iron and its biological relationship regarding iron metabolism.

SUMMARY

(1) The amount of iron liberated from the same sample of duck's egg-yolk by the action of sodium hydrosulphite, thiolacetic acid or sodium pyro-

phosphate is the same and it agrees fairly with the quantity of iron obtained after ashing. The trichloroacetic acid control gives negative test for iron.

(2) But in the case of fish tissue, the iron value obtained by the reduction with sodium hydrosulphite at pH 5.5 is less than that obtained by thiolacetic acid, but if the sodium hydrosulphite is added after treatment of the tissue with 8% acetic acid by Hill's method as modified by us (1), then the value becomes nearly equal to that obtained with thiolacetic acid. Treatment with sodium pyrophosphate gives only 30 to 40% of the iron in this case. Trichloroacetic acid extract of the control gives the test for iron, the amount being very small. Moreover, the amount of iron obtained after ashing is always greater than those obtained by any of the above methods. These observations indicate that, apart from the hæmatin content of fish tissue which does not exist in eggs, the forms in which the ionisable iron is present, are different in fish and egg.

(3) Ferric iron forms complexes with fish and egg but the ferrous iron does not do so.

(4) Enzymatic hydrolysis of fish and egg does not liberate any iron in a form, extractable by trichloroacetic acid.

(5) Iron added to egg-yolk cannot be recovered by enzymic hydrolysis, whereas in fish, added iron can be quantitatively recovered from the hydrolysate. This indicates that added iron forms perhaps an iron-protein complex in fish but not in eggs.

(6) Enzymic hydrolysis and increase in acidity do not increase the iron value of egg as in the case of fish and other animal tissues.

(7) Ascorbic acid can liberate some quantity of the iron in both egg and fish, but not as much as sodium hydrosulphite or thiolacetic acid under our condition of experiment.

(8) Thirty to forty per cent of the so-called available iron is present in a very stable combination which resists the action of pepsin and hydrochloric acid at pH 2.0.

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Letters to the Editor

My attention has been drawn to an article entitled "Some observations on the mustard oil—argemone oil theory of epidemic dropsy" by Mr. S. N. Sarkar in the March, 1941 issue of your journal. The argument developed by the author appears to be as follows:—

1. A comparison of the amounts of argemone mexicana oil contained in mixtures of mustard oil which, at the highest estimate, were able to give rise to symptoms of the disease in man in experiments conducted by Chopra *et al* (Ind. Med. Gaz., Vol. 74, p. 193) and the estimated amounts according to Pasricha's (Ind. Jour. Med. Res., Vol. 27, p. 951) calculation shows a great disparity between the two, the latter being many times more than the former.

2. A similar comparison of the amounts of epidemiologically incriminated oil which caused disease in human experiments with the estimated amounts worked out according to Pasricha's calculation leads to the hypothesis that the toxic oil obtained from the field must have contained about 50% of argemone oil or else the mustard oil—argemone oil theory would be open to doubt. Since the author is unable to accept such a high proportion of adulteration the latter inference would be logical. The hypothesis suggested is that the supposed toxicity might reside in the mustard oil itself.

To begin with one fails to see why in suggesting the search for the supposed toxic substance in *mustard oil itself* the facts concerning the mustard oil control groups employed in the feeding experiments have been overlooked. Further it should be remembered that the mustard oil used in the control groups was the same as used by the whole jail population none of whom suffered from any symptoms during and immediately following the experiments. This argument applies to both sets of experiments namely, by Chopra *et al* and by Lal *et al*. The suggestion for searching for the toxic substance in the oil itself does not therefore appear to be anything but entirely barren.

The rest of the argument rests on the hypothesis that the estimate of the amount of argemone oil required to cause symptoms of epidemic dropsy

in man given by Pasricha namely 0.88 cc. per 100 gms. of body weight is correct and since there is a great disparity between these and the experimental results the latter are incorrect or are capable of an alternate explanation. Let us examine the basis of Pasricha's estimate. The article in question deals wholly with experimental work on animals using mixture of argemone oil unheated and heated at different temperatures, the test of toxicity being loss of body weight of 10% or more. As regards man it is simply mentioned in a single sentence that criterion of toxicity is the onset of aches and pains in the body and the development of oedema but no experimental data for calculating the amount of argemone oil required to produce these symptoms is given. Yet the author takes his stand on Pasricha's estimate to throw doubt on the interpretation of experimental data. Isn't it an extraordinary argument?

The fact is that the results of a subsequent human experiment shortly to be published are in conformity with the estimate of the toxic dose of argemone oil described by the author from Chopra's paper.

In the final paragraph it has been claimed that the author has been able to "obtain a sample of mustard oil in the laboratory by a process excluding the possibility of the presence of argemone mexicana seeds", resembling in physical and chemical properties of the potent mustard oil obtained from the affected area. I am eagerly looking forward to a subsequent communication in which the author promises to give details of the preparation of the particular sample of mustard oil. In any case, this finding even if it is accepted at its face value does not necessarily invalidate the mustard oil— argemone oil theory of the causation of epidemic dropsy.

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I have read with interest the statement of Dr. R. B. Lal in connection with my paper "Some observations on the Mustard Oil—Argemone Oil Theory of Epidemic Dropsy" published in the March, 1941 issue of your journal.

Regarding Dr. Lal's comment in the fourth para that the present author has overlooked the fact that pure jail mustard oil does not produce any symptoms of epidemic dropsy and as such the search for toxic substance in the mustard oil in the absence of argemone oil is nothing but barren, I am to

say that this fact has not been overlooked by me. What I mean is that a sample of mustard oil responding to all the physical and chemical tests suggested for the epidemiologically incriminated mustard oil by R. B. Lal, S. P. Mookherjee, S. C. Roy and G. Sankaran (Ind. Jour. Med. Res., 27, 209) can be obtained from mustard seeds free from *Argemone mexicana* seeds by a special treatment. I hope to enlighten Dr. Lal on this matter, if possible, in the next issue of this journal.

In the fifth para Dr. Lal has laid the whole blame on Major Pasricha's shoulders suggesting in a round-about way that all his (Pasricha's) statements are not accurate. The paper containing these statements was published in April 1940 but no contradiction came from Dr. Lal though some of them appeared to him to be incorrect.

Dr. Lal has stated that his results on human experiments shortly to be published are in conformity with those of Chopra *et al* (R. N. Chopra, C. L. Pasricha, R. K. Goyal, S. Lal and A. K. Sen ; Ind. Med. Gaz., 74, 193). In this particular paper Major Pasricha is associated as the second author whereas in the other paper (C. L. Pasricha, S. Lal and K. Banerjee ; Ind. Jour. Med. Res., 27, 951) published at later date he is the sonior author. It is a general convention to accept later results as more correct but it seems that Dr. Lal wants to follow a different procedure. The same worker Major Pasricha is associated in two papers but Dr. Lal will accept his earlier result and will accuse him for his later result! If the supporters of the argemone theory are themselves not in agreement with each other in their findings then surely it is no fault of mine if I simply point it out.

Recently Mr. A. C. Bose who is working with me on the effect of feeding argemone oil to animals has obtained some interesting results. A group of six growing albino rats (initial combined body weight—461.5 g.) were each given a daily dose of 0.5 cc. of pure and freshly expressed argemone oil. The body weight began to decrease and reached a minimum (396.5 g.) ; the general appearance of rats was also somewhat sickly and there was a marked loss of hair. It has been suggested by many of the supporters of argemone theory that argemone oil toxin has a cumulative effect. The above experiment was, therefore, continued but it was observed that the body weight instead of going down further *began to increase* together with the resumption of hair growth. This increase was maintained and after ingestion of 15.5 cc. of oil the combined body weight was 575.5 g.—much higher than the initial. The feeding of argemone oil was continued for forty days and after that these animals were kept under observation for a fairly long time but nothing untoward could be noticed. There was no mortality in this group. A series of experiments are being carried out with different doses of argemone oil and also with different animals.

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The above experiment shows that argemone oil is not very toxic to animals and in the dose given it has no cumulative effect at least in rats. If it had a cumulative effect then the drop in the body weight would have been maintained resulting in the death of the animals. I wonder what explanation can be given of this finding consistent with the argemone theory.

It is well known that various theories of epidemic dropsy have been proposed from time to time but none has, as yet, stood the test of time. In dealing with this peculiar problem we should, therefore, take into consideration all possible factors. A very crucial test which could have set all doubts against argemone theory at rest has not yet been done. It is the isolation of active principles from argemone oil and epidemiologically incriminated mustard oil, to show that these substances are chemically identical and to prove further that they have identical physiological properties. So long as these points are not established the argemone contaminated mustard oil theory of the causation of Epidemic Dropsy cannot be considered final and all other possibilities ruled out.

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AGGLUTINATION REACTION IN EXPERIMENTAL ANIMALS
IN RESPONSE TO *PLASMODIUM KNOWLESI* ANTIGEN

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Agglutination reaction in monkey malaria has been described by Eaton (1) in 1938. The immune sera were obtained from monkeys with chronic infection and super-infection with *P. knowlesi*. The antigen used for agglutination test was prepared from the infected blood of monkeys collected during acute phase of infection with *P. knowlesi*. It has been shown that the immune sera agglutinated only the red blood cells infected with mature forms of parasites but not the non-infected red cells nor the red cells infected with ring forms of parasites. Agglutination of malaria parasites freed from red blood cells by these sera was also noted. These agglutinable antigens of *P. knowlesi* were found to be extremely labile. Only the fresh preparations were sensitive to immune sera. This agglutination reaction was found to be strictly species specific. Antigen of *P. knowlesi* was agglutinated with immune sera from monkeys infected with *P. knowlesi* but not with *P. inui*. Singh and Singh (2) recently corroborated the findings of Eaton and observed that *P. knowlesi* antigens are not agglutinated with sera of monkeys chronically infected with *P. inui* and *P. cynomolgi*. Symogyi (3) also observed that

serum of monkeys chronically infected with *P. knowlesi* is capable of agglutinating the infected R.B.C., provided 5 to 10% of the corpuscles contained mature parasites. He also noted that the agglutinating property of the antigen could be maintained up to 3 weeks by preserving the blood in physiological saline, to which 2% formalin had been added. Eaton and Coggeshall (4) immunised rabbits with injections of living or dead *P. knowlesi* and found that specific agglutinating antibodies could not be demonstrated in their sera. They (5) also failed to obtain agglutinating antibodies in the sera of monkeys artificially immunised by injections of dead *P. knowlesi* antigens, although complement-fixation tests with sera of both the immunised monkeys and rabbits were positive.

The present work was undertaken in connection with the investigations on the antibody response in experimental animals, which have been actively immunised with antigens of *P. knowlesi*. The first part of the investigation comprising work on complement-fixation with *P. knowlesi* antigen has been published (6). In the present paper antibody response in the form of specific agglutinins against *P. knowlesi* is recorded.

EXPERIMENTAL

Antigen.—Monkeys at the height of acute infection with *P. knowlesi* were bled from the heart, when the smear examination showed a very high percentage of red cells infected with *mature* parasites. The blood was defibrinated by shaking with sterile glass beads. The blood was then poured into tall glass tubes and allowed to settle. Within a short time the non-infected red cells settled down and the grey upper layer consisting mostly of cells infected with mature parasites was pipetted off and centrifuged at low speed. The plasma was thrown off and the infected R. B. C. was washed in saline. It was suspended in sterile physiological saline in proportion of *1 in 100*. This emulsion always contained some percentage of non-infected red cells, which acted as control. In true positive test the parasitised R. B. C. agglomerated while the non-infected R. B. C. remained discrete, whereas in false positive reactions both infected as well as non-infected cells agglutinated. Freshly prepared antigens were used. It was observed that, on keeping, the infected red cells were spontaneously hæmolysed. The hæmolysis of the infected R. B. C. became well marked before 24 hours, whereas with non-infected cells it was comparatively delayed. After hæmolysis the malaria parasites in many instances tended to auto-agglutinate and so became unsuitable for agglutination test. These malaria parasites retained their ability to agglutinate for some time under the influence of immune serum, although they as well as the parasitised red cells tended rapidly to lose their agglutinating power. It is, therefore, always advisable to utilise fresh antigens for agglutination test. Attempts to maintain the agglutinability of the antigens by using formalin or normal monkey serum proved unsatisfactory.

IMMUNE SERUM

Blood was collected from the femoral vein of monkeys. The serum separated from the blood was put to a bath at 56° for half-an-hour and kept in ice-box for future use.

Serum from monkeys chronically infected with *P. knowlesi* was tested for their agglutinating properties. The period of infection varied between 1 and 9 months. Three monkeys with chronic infection with *P. knowlesi* were super-infected by intravenous injections of 3 cc. of blood from monkeys heavily infected with *P. knowlesi*. Each received 8 such weekly injections. Their blood was then tested for agglutination titre and kept in ice-box. After one and a half months since the last injection fresh samples of blood were collected from the same monkeys and the two samples of blood were then titrated for their comparative rise or fall of agglutinins after the stoppage of super-infection. Each of the monkeys was then administered one further dose of 5 cc. of heavily infected blood intravenously and its effect on the agglutination titre was noted.

Four monkeys were artificially immunised by antigens prepared from parasitised red cells. These immunising antigens were prepared as follows:

IMMUNISING ANTIGEN I—INFECTED R. B. C. IN SALINE

Infected red cells containing mature *P. knowlesi* were collected at the terminal phase of acute infection in monkeys. They were washed twice with sterile physiological saline and resuspended in dilution of 1 in 10 in physiological saline containing 0.4% formalin. It was then put into ampoules or rubber-capped phials tested for sterility and kept in a refrigerator for future use. Sufficient time was allowed to pass to ensure the death of the parasites. The red cells both infected and non-infected, were completely hæmolysed in course of time.

IMMUNISING ANTIGEN II—ANTIGEN BY FREEZING AND THAWING

The washed parasitised R. B. C. was resuspended in the proportion of 1 in 10 in sterile physiological saline and hæmolysed by alternate freezing and thawing 3 or 4 times. It was then centrifuged and the parasitic deposit was washed twice with sterile saline and resuspended in the proportion of 1 in 10 in either 0.5% phenol or 0.4% formal saline. It was then sealed in glass ampoules or rubber-capped phials and kept in a refrigerator after sterility test.

IMMUNISING ANTIGEN III—ANTIGEN BY HÆMOLYSIN-CELL-COMPLEMENT SYSTEM

Here the hæmolysis of the infected R. B. C. is performed by the addition of anti-monkey R. B. C. serum and complement. The details of this technique

have been described in our previous paper (6). The parasites freed by this method were then washed and resuspended as in Antigen II.

Monkeys were immunised by injecting the antigens intravenously at an interval of 5 to 7 days. The dose varied between 0.5 and 10 cc. It may be seen that as each of these immunising antigens contains 10% emulsion of either infected R. B. C. or packed parasitic substance, the number of parasites per injection was fairly high, varying roughly between one and five hundred thousand millions. Monkeys Nos. 68, 69 and 71 each received in course of 14 injections covering over a period of 3 months a total dosage of 36 cc. of 10% packed parasitic substance or infected R. B. C.

TEST PROPER

The technique of the agglutination test as described by Eaton (1) was adopted in general, with certain modifications which were found convenient.

Test tubes used in the present series of tests were those which are usually utilised for standard Kahn test. They are 1 cm. in inner diameter and 7.5 cm. in length.

Serum with unknown titre was used undiluted and progressively diluted in saline in dilutions of 1 in 2, 4, 8, 16, and so on. With known sera of high titre usually the progressive dilution from tube to tube was 4 times. 0.3 cc. of serum diluted or undiluted was taken in each tube. And an equal volume of agglutinating antigen (1% emulsion) was then added. For each serum a series of tubes was arranged. In each was taken 0.3 cc. saline. When the progressive dilution was 4 times from tube to tube, 0.1 cc. of serum was added in the first tube making the dilution 1 in 4. 0.1 cc. from the first tube was transferred to the second tube, in which the dilution of serum became 1 in 16. In subsequent tubes a similar method was adopted. When the progression of dilution was to be made double from tube to tube, 0.3 cc. of serum was used instead of 0.1 cc. and the same quantity was transferred from the first to the second tube. It may be noted here that the final mixture contained equal volumes of diluted serum and antigen. The actual dilution of serum was therefore double the strength of what is recorded in the table.

In control tubes normal monkey serum and saline were used instead of the serum to be tested. The mixture of anti-serum and antigen was then mixed by shaking and the tubes were left at room temperature for 2 hours. During the first 15 minutes the tubes were shaken gently 3 or 4 times. After 2 hours the reading was taken. The naked eye appearance was first noted. Microscopic reading was also taken to confirm macroscopic observations and eliminate false positive reactions.

In truly positive reactions the parasitised red cells, particularly those containing mature parasites, agglomerated leaving the non-infected cells as also those containing immature parasites free and discrete. In a false positive

reaction both infected as well as non-infected red blood cells agglutinated. When the reaction was strongly positive big granules settled at the bottom of the tube and the clumps did not break up by gentle shaking but were clearly visible in suspension. This reaction is represented as + + +. On microscopic examination it was found that the clumps of infected R. B. C. were big and almost all the red cells containing mature parasites agglomerated. When the reaction was moderately positive, the granules at the bottom of the tube were comparatively smaller. On gentle shaking these were visible in the solution. On microscopic examination the clumps of infected red cells were found to be fairly big. The number of non-agglutinated parasitised red cells was comparatively few. This reaction is represented as + + . Weakly positive reactions are marked as +. Granules at the bottom of the tube were still smaller but distinctly visible. Microscopic examinations revealed that the clumps were smaller and non-agglutinated parasitised red cells were present in fair number.

In cases of very weakly positive reactions the granules were not clearly visible to the naked eye. On shaking it formed uniform emulsions, where agglomeration was not seen. But when the undisturbed sediment was put on a slide with a cover glass definite agglomeration of the malaria parasites or infected red cells could be observed. The clumps consisted of fewer malaria parasites, while most of the infected red cells were free and discrete. This reaction is represented as $\pm$. When the test was negative (−) both non-infected and infected red blood cells were free and discrete. All the red cells settled down at the bottom of the tube as a compact mass. A pseudo-positive reaction was read when both infected as well as non-infected red cells agglutinated. This reaction was due to the presence of anti-monkey R.B.C. agglutinin.

ABSORPTION OF AGGLUTININS

Attempts were made to absorb agglutinins from the agglutinating sera. Sera with low agglutination titre were diluted 1 in 2 with sterile physiological saline, while those with higher titre were diluted 1 in 4 or 1 in 8. Two cc. of diluted serum were then taken in each of a series of 4 sterile test tubes. In the first tube were added 2 cc. of washed parasitised R.B.C. ; in the second tube were added 2 cc. of non-infected R.B.C. from a normal monkey, and in the third tube the same quantity cell-free packed parasitic substances. These parasitic substances were the same as immunising Antigens Nos. II and III. The fourth tube was kept for control. The tubes were then shaken and the mixtures were kept at room temperature for 2 hours and then overnight in ice-chest. They were then centrifuged and the supernatant fluids were separated and tested for their titre of agglutinins.

TABLE I

Showing the titre of agglutinin in sera of monkeys with chronic infection with P. knowlesi.

Serum of monkey No.	Periods of infection.	Highest titre of agglutination.	Lot no. of antigen.
99	1 month 3 days	32	112
	1½ months	64	,,
60	2½ months	1024	,,
	3½ months	2048	,,
65	2½ months	1024	,,
	3½ months	4096	,,
16	9 months	256	,,

It can be seen from Table I that monkey No. 99 with comparatively recent infection showed lower titre of agglutinin. Monkeys no. 60 and 65 developed comparatively higher titre of agglutinins in their sera. But no. 16 with the longest period of infection showed comparatively very low titre of 1 in 256 only. This may be due to individual variations in antibody response from animal to animal, a fact which had been observed by present authors (6) in connection with complement-fixation reaction. Individual variations were also recorded by Eaton in connection with agglutination reactions. It may be mentioned here that M. 16 was infected by a strain of P. knowlesi, which was collected from a source other than that of the strain by which M. 60, 65, 99 and also the monkeys from whose blood were prepared the agglutinating antigens were infected. Whether these two strains were identical or different, could not be traced. Furthermore whether there exists any variation in the power of stimulating antibody formation by different strains, or whether antigen prepared from one strain is less sensitive to the antibody whose formation has been stimulated by another strain of P knowlesi has not been worked out. In the absence of such evidence it may be accepted as an instance of comparatively poor response in agglutinin although the period of infection was the longest.

Sera of 12 normal or non-infected monkeys were tested, but in no case agglutinin could be detected. Agglutinins were also absent in the sera of 12 monkeys during acute infection, even up to the terminal phase which usually came before the lapse of 10-12 days from the injection of the infecting dose of malaria parasites. Traces of agglutinin have been recorded after 2 weeks of infection in treated cases. In the case of M. 99 the titre was 1 in 64 on the 33rd day of infection, whereas it was found as high as 1024 at the end of 2½ months in case of M. 60 and 65. The titre rose up to 2048 in M. 60

and 4096 in case of M. 65 at the end of 3½ months. It appears, therefore, that when the acute infection subsides the antibodies begin to appear in blood and their titre rises comparatively rapidly with the periodic parasitic relapses, until the monkey develops sufficiently strong defensive mechanism when the infection becomes comparatively latent, with very few relapses, although parasite count in the peripheral blood shows some irregular variations from time to time.

EFFECT OF SUPER-INFECTION

The comparative rôle of super-infection and the duration of infection on the rise of titre of agglutinin had been left undecided by former workers. While in the series of experiments of Eaton (1) the titre of agglutinins in the sera of super-infected monkeys was generally higher than those of chronically infected monkeys, it was doubted whether it was the chronicity of infection or the super-infection, which caused the rise in titre of agglutinins. It may be noted that the duration of infection in the super-infected group was generally longer than in the chronically infected group. Owing to the variability in the titre of antibody response in animals, difficulties had to be faced in coming to a conclusion. It had been suggested by Eaton that "further work with a larger series of monkeys will be necessary before the relation . . . . . can be definitely established". The same conclusion was also drawn by Singh and Singh (2). In order to overcome the difficulties due to individual variations, we studied the changes in titre in the same monkeys from time to time. Samples of sera from each of the three super-infected monkeys were collected 10 days after injections of a course of 8 weekly super-infecting doses and then one and a half months after the stoppage of super-infection. Then again a single dose of 5 cc. of heavily infected blood was injected into each of the monkeys and their sera were collected after a further period of 10 days. , These three samples of sera from each of the monkeys were titrated against single antigen on the same day. The result is given in Table II

TABLE II
Showing the effect of super-infection.

Monkey No.	Period of infection at the end of first course of super-infection	Agglutination titre 10 days after 8 super-infecting doses.	One and a half months after the 8th dose of super-infection.	10 days after the 9th dose of super-infection.
25	3 months 8 days	2048	1024	4096
43	3 months 5 days	1024	256	4096
57C	3 months 4 days	1024	1024	1024

It will be seen from the above table that in 2 out of the 3 monkeys there was a definite fall in agglutinin titre one and a half months after the super-infection had been stopped. The titre again rose abruptly following an injection of a single super-infecting dose into both of them. It is, therefore, clear that when super-infection was stopped there was a fall in titre although the duration of infection increased. Again, the definite way by which the agglutinin titre went up following a single super-infecting dose appears to make the rôle of super-infection and duration of infection comparatively clearer to understand.

It may be mentioned here that M. 16, whose serum showed a titre of 1 in 256, was super-infected with 2 cc. of blood with a strain of *P. knowlesi* used for the preparation of agglutinating antigen. There was some rise in the parasite count which soon subsided. Blood collected on the 10th day after super-infection showed a titre of 1 in 1024.

It may also be noticed that the maximum titre of agglutinins in the sera of monkeys in the present series of experiments was comparatively higher than that recorded by previous workers. This can partly be explained by the fact that we used 0.3 cc. of serum instead of 0.2 cc. for the test. Other alterations in the technique in the test might also be remembered. Monkeys used in these experiments belonged to *Silenus Rhesus* group.

TABLE III

Showing the effect of artificial immunisation of monkeys by injection of dead P. knowlesi *antigen.*

Serum of monkey No.	Lot no. Immunising antigen.	Method of preparation of the antigen.	No. of injection.	Period of immunisation.	Total quantity of antigen.	Titre of agglutination.
M-104	80 and 106	Freezing and thawing.	4	23 days	23 cc.	nil.
M-68	55 (i) and 61 (ii)	Hæmolysin-cell-comp. system.	14	3½ months	36 cc.	1 in 64
M-69	55 (ii) and 72	Freezing and thawing.	14	3½ months	36 cc.	with undiluted serum.
M-71	55 (iii) and 64	Formalised emulsion of infected R.B.C.	14	3½ months	36 cc.	nil.

It will be seen from the above table that of the 4 monkeys artificially immunised with antigens consisting of killed *P. knowlesi,* M. 68, 69 and 71 received in 14 injections spread over a period of 3½ months 36 cc. of 10% emulsion of packed parasitic substance or R. B. C. infected with mature parasites. Whereas M. 104 received only 4 injections in 23 days of 23 cc. 10% emulsion of packed parasitic substance. The dose of antigen injected in each of the 4 monkeys was fairly high. And the period of immunisation at least in the cases of M-68, 69 and 71 was reasonably long. But of the 4 monkeys only M. 68 showed some fair titre of agglutinins in its serum after the full course of immunisation. And while the agglutinins in the serum of M. 69 were present only in'traces, those in the sera of M. 71 and 104 were totally absent. Whether this result was due to the variation in the power of antibody response of monkeys or in the quality of the antigens could not be determined in the small series of experiments.

EFFECT OF ABSORPTION OF AGGLUTININ

Sera of 3 monkeys were absorbed with normal R. B. C., parasitised R. B. C. and cell-free malaria parasites. The effect of absorption is given below.

TABLE IV

Showing the effect of absorption of agglutinins.

Serum of monkey no.	1st tube with parasitised R.B.C.*	2nd tube with malaria parasites.*	3rd tube with normal R.B.C.*	4th tube not absorbed.*
57C (super-infected)	1 in 16	1 in 4	1 in 1024	1 in 1024
68 (immunised by antigen)	1 in 2	1 in 2	1 in 64	1 in 64
60 (chronic infection)	1 in 16	1 in 16	1 in 1024	1 in 2048

*Figures show maximum titre in each tube.

It will be seen in the above table that from the sera of monkeys of both chronic infection and super-infection as well as of one immunised by antigens of dead *P. knowlesi,* agglutinins could be absorbed by fresh parasitised R.B.C., as well as cell-free malaria antigens. But normal R.B.C.

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failed in that respect. It may be mentioned here that the parasitised R.B.C. used for absorption test were collected on the same date on which the tests were performed ; the malaria antigens used for the same purposes were one to three months old. It is therefore interesting to note that although old antigens proved absolutely useless for use in agglutination test, they could after injection give rise to the production of agglutinins and could also absorb agglutinins from the positive sera. This absorbing property was not due to the non-specific R.B.C. factor, because the latter alone failed to absorb the agglutinins.

DISCUSSION

Agglutinating antibodies begin to appear in the blood of monkeys soon after the acute phase of infection passes off as the result of treatment. The titre of agglutinin rises as the infection becomes chronic. The titre appears to increase progressively with the duration of infection. Super-infection enhances the titre and it appears that the titre falls to some extent after the super-infection is discontinued. A single super-infecting dose by this time again effects a rise in titre. It may be noted that the appearance of rise and fall of the agglutinating antibodies in the sera of monkeys may be correlated roughly with the anti-parasitic immunity, which, on the other hand, is somehow inversely proportional to the parasite count in the peripheral blood of the monkeys For example, soon after malaria parasites appear in the peripheral blood, usually 4-5 days after injection of the infecting dose of P. knowlesi, the parasite count rapidly rises. If the animal is kept untreated the parasites increase very rapidly and ultimately in another 4-6 days' time overwhelm the host, which cannot put forward any anti-parasitic resistance. Agglutinating antibodies are found to be totally absent uptil this time. If the monkeys are treated during the acute phase they gradually develop workable immunity in a short time and the rise in the parasite count is controlled. Gradually with the rise of immunity the parasite count begins to fall. Along with this anti-parasitic immunity other antibodies begin to appear in the blood, although they may not be detectable by laboratory tests until some time passes. Eaton has demonstrated agglutinating antibodies on the 15th day of injection with P. knowlesi in quinine treated cases. The titre of demonstrable antibodies rises as the period of infection increases, while the parasite count in the peripheral blood falls persistently. Coggeshall and Kumm (7) and subsequently others have demonstrated protective antibodies in the sera of monkeys chronically infected with P. knowlesi. Their sera have been shown to have prophylactic and curative properties on other monkeys infected with P. knowlesi. Coggeshall and Eaton (9) have observed that complement-fixing antibodies develop in sera of monkeys during sub-acute and chronic stages of infection with P. knowlesi.

Hyper-immunisation by super-infection has been found to enhance the development of antibodies. It has been observed in connection with the present series of experiments that after each injection of the first few super-infecting doses the monkeys developed parasitic relapse, with temporary rise in parasite count but during the course of subsequent injections parasites practically disappeared from the peripheral blood and the infection became latent. With this rise in the anti-parasitic immunity there has been noted simultaneous increase in the titre of other antibodies in the blood. Coggeshall and Kumm (8) have demonstrated an increase in the titre of protective antibodies in chronically infected monkeys as a result of super-infection. Coggeshall and Eaton (9) have recorded the presence of direct relation between super-infection and enhancement in the titre of complement-fixing antibodies. Similar relation has also been recorded with agglutinating antibodies in the present series of experiments.

It would, therefore, appear that the development, rise and fall of these antibodies. *e.g.*, anti-parasitic, protective, agglutinating and complement-fixing antibodies run parallel to each other, although it is possible that a particular monkey may show high titre of one antibody, while that of others may be comparatively low or even absent.

The question, however, becomes more complicated when one takes into consideration the artificial immunisation of laboratory animals with antigens of *P. knowlesi*. It has been observed by Eaton and Coggeshall (5) that monkeys immunised artificially by antigens from dead *P. knowlesi* showed in their sera complement-fixing antibodies in fair titre but in no case agglutinating antibodies could be detected by them in artificially immunised monkeys. Such monkeys in their experiments also failed to demonstrate protective antibodies in their sera. In a previous paper (6) by the present authors complement-fixing antibodies in the sera of artificially immunised monkeys and rabbits have been recorded. In the present series of experiments it has also been possible to demonstrate positive agglutination reactions in the serum of 2 monkeys immunised by injections of malaria vaccines, although much bigger doses and prolonged immunisation were necessary to achieve success. But these big doses do not appear to be too much, when one takes into consideration the total amount of malaria parasites, which is thrown into circulation of an infected monkey up to the time of appearance of agglutinins in its serum.

But at the present state of our knowledge regarding serum antibodies in malaria, it appears that more work has to be carried out before any conclusive opinion regarding the mechanism of production and significance of these antibodies, and particularly, those that are concerned with anti-parasitic and protective immunity can be formed.

SUMMARY

1. Agglutinating antibodies appear in the sera of monkeys infected with *P. knowlesi* after the acute phase of infection passes off. The titre of agglutinin increases with the duration of infection.

2. Super-infection causes a rise in the titre of agglutinins, at least in some cases.

3. It has been possible by artificial immunisation of monkeys with antigens of dead *P. knowlesi* to produce agglutinins in their serum.

4. Absorption of the specific agglutinins from the immune sera could be obtained by parasitised R.B.C. as well as cell-free parasites.

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OBSERVATIONS ON THE SPLITTING OF THE COMBINED ASCORBIC ACID OF URINE

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It has been shown by Banerjee (1) that normal human urine contains ascorbic acid in dehydro- and combined forms besides free ascorbic acid. Similar observations have been reported by Banerjee, Sen and Guha (2, 3) in their studies on tuberculous urine. In their methods of estimation they have used hot hydrogen sulphide to split up combined ascorbic acid. Scarborough and Stewart (4) have shown that prolonged treatment with hydrogen sulphide increases the indophenol reducing power of urine and according to their view this is a simple case of hydrolysis as they have also observed an increased value of indophenol reducing substance on continued heating of urine acidified with glacial acetic acid in a current of carbon dioxide. Similar increase in indophenol reducing power of urine by boiling with 5% sulphuric acid for 15 minutes in a current of carbon dioxide has been reported by van Eekelen (5). Many investigators have found an increased value of ascorbic acid on heating an aqueous plant extract on water-bath in a current of carbon dioxide, and Guha and Pal (6) have shown that a considerable portion of ascorbigen is split up when an aqueous extract of cabbage containing 0.2% hydrochloric acid is allowed to stand at 31° for 1 hour. On the other hand, Banerjee, Sen and Guha (3) have used 5% sulphuric acid (on the volume of urine) to preserve urine for 24 hours in their study of urinary excretion of combined ascorbic acid in pulmonary tuberculosis. It would appear, therefore, that combined ascorbic acid in urine is more stable than ascorbigen found in plant kingdom. There appeared to be some peculiar features regarding the splitting of combined ascorbic acid of urine in our early experiments. It seemed to split more readily if the urine was heated in a current of hydrogen sulphide for 15 minutes than if it was heated in a current of carbon dioxide for one hour. It was considered desirable to investigate more fully the splitting of combined ascorbic acid of urine under different conditions of hydrogen ion concentration and temperature and in currents of hydrogen sulphide and carbon dioxide in order to throw more light on the behaviour of this substance.

EXPERIMENTAL

I. In our preliminary experiments we failed to observe any increase of ascorbic acid value by heating the urine acidified with glacial acetic acid (1% by volume) on water-bath in a current of carbon dioxide for one hour ; but treatment with hot and cold hydrogen sulphide revealed the presence of combined ascorbic acid in urine. This difference in behaviour was further investigated in the following experiments.

Freshly passed urine was acidified with glacial acetic acid (1 cc. per 100 cc of urine) and divided in four aliquots, which were treated severally as follows :

(*a*) The urine was immediately titrated against 2:6-dichlorophenol-indophenol and correction was made for interfering substances by treatment with ascorbic acid oxidase in the usual manner (1).

(*b*) The urine was heated on the water-bath in a current of carbon dioxide for one hour and titrated as before. Into one portion of the carbon dioxide treated urine, hydrogen sulphide was passed in the cold condition for half an hour and the sulphide was removed by a current of coal gas, purified by bubbling through chromic acid solution. Coal gas so treated was found in control experiments not to affect the ascorbic acid value. After removal of hydrogen sulphide the solution was titrated against the dye.

(*c*) The urine was treated with hydrogen sulphide in the cold for half an hour and after removal of hydrogen sulphide it was titrated as before.

(*d*) The urine was treated with hydrogen sulphide successively for 5 minutes in the cold, 15 minutes on the boiling water-bath and for another 10 minutes in the cold condition. After removal of hydrogen sulphide the solution was titrated against the dye.

TABLE I.
Mg. of ascorbic acid in 100 cc. of urine.

Sample of urine.	Original urine.	CO_2-treated urine.		H_2S.-treated urine.	
		Before treatment with H_2S.	After treatment with H_2S.	Cold H_2S.	Hot H_2S.
1.	5.436	5.436	6.748	6.748	7.886
2.	0.264	0.264	0.264	0.264	0.492
3.	0.855	0.855	0.935	0.927	1.335
4.	1.135	1.135	1.777	1.777	1.948
5.	1.065	1.064	1.185	1.185	1.504
6.	1.033	1.033	1.115	1.115	1.278
7.	2.146	2.146	2.446	2.446	3.680
8.	3.803	3.803	4.093	4.093	5.198

In every case ascorbic acid oxidase treatment was resorted to in order to eliminate nonspecific reducing substances. The results are given in Table I.

From the above results it is evident that combined ascorbic acid is not split up by heating urine acidified with acetic acid in an atmosphere of carbon dioxide for one hour but 15 minutes' treatment with hydrogen sulphide is sufficient for the purpose.

II. In the next set of experiments sulphuric acid was used instead of acetic acid and the pH of urine was adjusted to definite values to see what effect hydrogen ion concentration had on the splitting of the combined ascorbic acid of urine. The fresh urine was first acidified with dilute sulphuric acid in order to stabilise ascorbic acid and then the pH was adjusted with dilute caustic soda solution. The acidified urine sample was divided into 4 aliquots, which were treated severally as follows:

(a) One portion was simply titrated against the dye.

(b) The second portion was heated in carbon dioxide atmosphere for one hour and titrated.

(c) The third portion was treated with cold H_2S for half an hour and, after removal of hydrogen sulphide, was titrated as usual.

(d) The fourth portion was treated with hydrogen sulphide for 5 minutes in the cold, for 15 minutes in the hot condition and again for 10 minutes in the cold. After removal of the sulphide, the solution was titrated as usual. In each case ascorbic acid oxidase was used to find the true ascorbic acid value. The results are given in Table II, in which the ascorbigen values as obtained by heating

TABLE II.
Mg. of ascorbic acid in 100 cc. of urine.

Sample of urine.	pH of urine.	Original value. (a)	CO_2-treated urine. (b)	Ascorbigen split by CO_2 (b—a)	Cold H_2S treated urine. (c)	Hot H_2S treated urine. (d)	Ascorbigen split by H_2S. (d—c)
1.	1.2	1.352	2.499	1.147	1.778	2.799	1.021
2.	1.2	0.436	1.066	0.630	0.598	1.191	0.593
3.	1.6	2.354	3.948	1.594	2.424	3.966	1.542
4.	1.6	4.178	5.597	1.419	4.178	5.597	1.419
5.	1.6	2.542	3.698	1.156	2.774	3.936	1.162
6.	2.0	5.258	6.260	1.002	5.590	7.780	1.190
7.	2.0	2.870	2.950	0.080	2.870	3.360	0.490
8.	2.0	1.646	2.390	0.744	2.016	3.072	1.056
9.	2.8	0.811	0.811	0.000	1.226	1.557	0.331
10.	2.8	3.803	3.803	0.000	4.093	5.198	1.105

in carbon dioxide for one hour and with hydrogen sulphide for 15 minutes are given in columns 5 and 8 respectively for comparison.

These results show that below pH 1.6, values of increased ascorbic acid obtained by carbon dioxide treatment for one hour are higher than those obtained by hot hydrogen sulphide treatment for 15 minutes. At pH 1.6 the two values are approximately identical, but above pH 1.6 carbon dioxide gives lower values and finally at pH 2.8 carbon dioxide is unable to split up combined ascorbic acid of urine.

III. In a third set of experiments we attempted to find, what effect, heating with hydrogen sulphide at different pH's would have on the splitting of the combined ascorbic acid of the same sample of pooled urine. Similar experiments were carried out regarding the effect of heating in carbon dioxide. The pooled urine was collected over dilute sulphuric acid in order to prevent oxidation of ascorbic acid, but it was observed that in course of adjustment of pH by dilute caustic soda, even when the pH is fairly low (2.0) there is loss of a small amount of ascorbic acid. The pooled urine was divided into three equal aliquots and treated as follows:

(1) The pH of one portion was adjusted to 1.6 by the addition of dilute caustic soda.
(2) The pH of another portion was adjusted to 2.0.
(A) The pH of the third portion was adjusted to 3.0.

After the adjustment of pH, all the three portions were diluted to a constant volume. Each was divided into four parts. One part was titrated to obtain the free ascorbic acid value and another part was immediately put in a current of carbon dioxide in order to prevent aerial oxidation and heated on the water-bath for one hour. In the other two portions dehydroascorbic acid and combined ascorbic acid were estimated by cold and hot hydrogen sulphide methods as described before. Table III gives the results obtained with 5 pooled samples of the urine.

It is clear from the above results that as the pH increases, splitting of combined ascorbic acid decreases both by CO_2 and by H_2S in the hot condition.

IV. Some experiments were conducted to see if 5% sulphuric acid, which is used as a preservative of urinary ascorbic acid, would completely stabilize ascorbic acid for 24 hours and whether it would tend to split the combined ascorbic acid of the urine. For this purpose fresh urine was collected over dilute sulphuric acid (1 : 4) and after measuring its volume a known amount of sulphuric acid (1 : 4) was added in order to make it 5%

TABLE III.
Mg. of ascorbic acid in 100 cc. of urine.

Sample of pooled urine.	pH adjusted to.	Original urine.	CO₂ treated urine.	Ascorbigen split by CO₂.	Cold H₂S treated urine.	Hot H₂S treated urine.	Ascorbigen split by H₂S.
1.	1.6	0.983	2.048	1.065	1.082	2.092	1.010
	2.0	0.849	1.047	0.198	1.082	1.693	0.611
	3.0	0.701	0.701	0.000	1.082	1.614	0.532
2.	1.6	1.388	2.458	1.070	1.576	2.528	0.952
	2.0	1.274	1.483	0.209	1.576	2.093	0.417
	3.0	1.014	1.014	0.000	1.576	1.924	0.348
3.	1.6	0.659	1.264	0.605	0.838	1.373	0.435
	2.0	0.453	0.659	0.206	0.838	1.128	0.290
	3.0	0.286	0.286	0.000	0.838	0.955	0.117
4.	1.6	0.613	1.385	0.772	0.755	1.372	0.517
	2.0	0.470	0.563	0.093	0.755	0.919	0.154
	3.0	0.381	0.381	0.000	0.755	0.919	0.154
5.	1.6	0.826	1.642	0.816	1.096	1.962	0.666
	2.0	0.706	0.894	0.168	1.096	1.748	0.452
	3.0	0.544	0.544	0.000	1.996	1.748	0.452

acid by volume. This urine was then divided into two parts ; one part was examined immediately for free, dehydro- and combined ascorbic acids and the other part was similarly examined after standing for 24 hours at room temperature (33.3°—34.4°). The results are shown in Table IV.

TABLE IV
Mg. of ascorbic acid in 100 cc. of urine.

Sample of urine.	Fresh urine.			After 24 hours.		
	Original urine.	Cold H₂S treated urine.	Hot H₂S treated urine.	Original urine.	Cold H₂S treated urine.	Hot H₂S treated urine.
1.	1.333	1.901	3.130	1.418	1.969	2.938
2.	2.134	2.316	5.020	2.354	3.588	4.216
3.	3.010	3.900	6.798	3.604	4.236	5.986
4.	2.836	4.266	8.354	3.356	4.540	6.580
5.	3.634	4.676	8.596	4.070	5.328	6.250
6.	3.554	4.376	10.556	4.810	6.010	8.968
7.	4.124	5.020	10.556	4.928	6.586	8.636
8.	2.021	2.171	4.150	2.133	2.337	3.851

The results show, that the free ascorbic acid obtained in the urine after standing for 24 hours at 33.3°—34.4° is actually more than that present in

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the fresh urine. The dehydroascorbic acid also increases on standing. The combined ascorbic acid, on the contrary, diminishes on standing. It would thus appear that some combined ascorbic acid splits up on allowing the acidified urine to stand for 24 hours at room temperature and it is apparently the ascorbic acid, that is thus released, which accounts for the increase of free and dehydroascorbic acids in the urine on standing. It is to be noted, however, that the "total" ascorbic acid diminishes on standing, which would indicate that a part of the ascorbic acid is irreversibly oxidised.

SUMMARY

1. If urine is acidified with 1% acetic acid and heated for 1 hour in carbon dioxide, the ascorbic acid value is not increased. This occurs, however, if heating is done in H_2S for 15 minutes.

2. If, however, the pH of the urine is 1.6, heating in carbon dioxide for one hour produces the same increase in ascorbic acid value as heating in H_2S for 15 minutes, showing that it is the low pH which is particularly important for the splitting of combined ascorbic acid. With increasing pH the splitting diminishes more rapidly in an atmosphere of carbon dioxide than of H_2S.

3. If urine containing 5% sulphuric acid by volume is allowed to stand for 24 hours at 33.3°—34.4° the values of free and dehydroascorbic acids increase, while that of combined ascorbic acid decreases. The value of total ascorbic acid also diminishes, showing the irreversible oxidation of a part of the ascorbic acid.

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AVAILABLE IRON IN FISH. PART III. FURTHER STUDIES ON THE NATURE OF NON-HAEMIN IRON IN FISH

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In a separate paper (1) we have reported that peptic hydrolysis of fish tissue leaves an insoluble residue, which contains 30 to 40% of the total ionisable iron of the tissue. It was of interest to investigate the nature of iron in this residue. The results of this work are presented in this communication.

EXPERIMENTAL

Methods for the isolation of the complex iron-bearing compound.—The edible portion of the fish under investigation (*Labeo rohita*) is cut into small cubes, the black skins of the cubes are separated from the fleshy portion and then finely minced. The minced fish (100 g.) is taken with 400 cc. of distilled water in a 1000 cc. flask. The mixture is well shaken and the *p*H adjusted to 2.0 with N/5 hydrochloric acid. To this are added 25 cc. of 0.4% pepsin solution and 6 to 7 drops of toluene and the mixture is then incubated at 37° for 24 hours. After incubation the hydrolysate is separated from the residue by centrifuging. The residue is made free from hydrochloric acid by repeated washing with water and centrifuging. The colourless residue is boiled with 90% alcohol and centrifuged again. It is then washed with a mixture of absolute alcohol and ether (1:1) and finally with ether and dried in vacuum.

Relation between the periods of peptic digestion of the raw fish and the percentage of iron-complex left behind.—In order to see whether the iron-bearing complex left behind after peptic digestion of the raw fish is of constant composition, the same sample of fish muscle-tissue is digested with pepsin solution for varying periods and in each case the percentage of iron-complex left behind is determined by weighing. The method adopted in this case is the same as described above. The results are given in Table I.

TABLE I.

Periods of peptic digestion.	Fresh fish meal.	Amount of the dried iron-complex per 100 g. fresh tissue.
24 hours.	75 g.	0.70 g.
48	75	0.69
72	75	0.78
96	75	0.73

From the above results, it is clear that within 24 hours the peptic hydrolysis is complete and the residue left behind resists further action. Tryptic hydrolysis could not be applied as alkali by itself has been found to liberate iron from the complex.

The dried iron complex on estimating by Hill's modified method for ionisable iron (2) and by ashing for total iron gives the following values, which are approximately equal showing that practically the whole of iron of this complex is ionisable.

Hill's method (in 8% acetic acid).	After ashing.
44.2 mg. per 100 g. of the dry iron-complex.	43.6 mg. per 100 g. of the dry iron-complex.

Estimation of iron in the original fresh raw fish sample from which the iron-complex has been prepared.—In order to estimate how much of the total ionisable iron is present as organic iron complex which resists the action of pepsin, estimations of iron have been carried out in presence of sodium hydrosulphite (in 8% acetic acid) and thiolacetic acid in the same sample of fish tissue from which the iron-complex has been prepared. The results per 100 g. of fresh tissue are given below:—

Iron in mg. % by Hill's modified method (in 8% acetic acid).	Iron in mg. % by thiolacetic acid.	Iron in mg. % obtained by ashing.
0.87	0.85	1.12

Therefore 100 g. of fresh fish tissue contain 1.12 mg. of total iron, of which 77% is ionisable. As 100 g. of the fresh tissue yield 0.73 g. of dried iron-complex, which contains 44.2 mg. ionisable iron per 100 g. of the iron-complex, about 37% of the ionisable iron of the fresh tissue is retained in the iron-complex obtained after peptic hydrolysis.

Solubilities of the iron-complex.—The substance is found to be insoluble in cold and boiling water, benzene, chloroform, acetone, dilute or strong mineral acids under ordinary conditions, ammonia, sodium carbonate solution, solutions of sodium chloride and ammonium sulphate of all concentrations. It is soluble in strong and dilute sodium or potassium hydroxide solutions at ordinary room temperature. On boiling with moderately strong mineral acids iron is liberated.

Chemistry of the iron-complex.—The following tests indicate that it is a nucleoprotein in combination with iron and copper.

(*a*) *Tests for proteins :* The substance responds to xanthoproteic and Millon's reactions.

(*b*) *Tests for carbohydrates :* A small portion of the substance is boiled with 10% sulphuric acid and the resulting solution gives positive Molisch and Bial's tests.

(*c*) *Test for phosphorus :* A small portion of the substance in a crucible is heated with five times its bulk of fusion mixture. It is then carefully fused until the resulting molten mass is colourless. It is cooled, dissolved in a little warm water, acidified with nitric acid and heated to about 65°. On addition of a few cc. of molybdate solution a yellow precipitate results. As the substance was obtained by peptic hydrolysis in an acid medium, the presence of acid soluble phosphorus in the substance is excluded. The above test, therefore, shows that it contains organically-bound phosphorus.

(*d*) *Test for purine bases:* A small portion of the substance is heated under reflux with 10N-hydrochloric acid for one hour. The resulting solution is then evaporated to dryness on water-bath with a little potassium chlorate. The yellowish residue is moistened with ammonium hydroxide solution and again warmed gently. A purplish red colour develops indicating the presence of purine bases.

(*e*) *Test for copper:* A small amount of the substance is digested with sulphuric and perchloric acid mixture. To the clear solution thus obtained 1 cc. of 4% sodium pyrophosphate solution is added followed by the addition of ammonia until slightly alkaline to litmus. Then on the addition of 2 cc. of sodium diethydithiocarbamate solution (2%) and 50 cc. of amyl alcohol, an intense yellow colouration develops showing the presence of copper. The copper present can also be extracted with 20% trichloroacetic acid indicating thereby that copper is present in a loose combination with nucleoprotein.

Determination of Fe : P : N *ratio in the iron-nucleoprotein complex.—* In order to test the purity of the substance the Fe, P and N ratios have been determined after different treatments as follows:

1. *Treatment with hot water :*

0.5 G. of the substance under investigation is heated with 10 cc. of water and then filtered hot. The filtrate and residue are evaporated to dryness and weighed separately. The filtrate contains only a minute quantity of material.

Estimation of Fe, P and N in the residue: The residue left behind after the treatment with hot water is weighed and taken in a small silica crucible and heated to a white ash. The ash is boiled with 10 N-hydrochloric acid and filtered. The solution is made up to 50 cc.; Fe and P are determind in aliquots. Nitrogen in a portion of the residue is determined by micro-Kjeldahl method. Similar estimations were carried out with the residue obtained from the filtrate.

2. *Treatment with 0.2% sodium hydroxide solution and subsequent precipitation with acetic acid and trichloroacetic acid mixture at pH 2.0.:*

One g. of the substance is heated with 20 cc. of 0.2% sodium hydroxide solution for 30 minutes. It is then filtered and the filtrate is adjusted to *p*H 2.0 with acetic and trichloroacetic acid mixture (1 cc. of glacial acetic acid and 1 cc. of 20% trichloroaetic acid) and centrifuged. The precipitate is washed with trichloroacetic acid (5%), and dried. Fe, P and N determinations are made both in the residue from caustic soda treatment and in the precipitate after treating the filtrate with trichloroacetic acid.

3. *Recombination of iron with the residue obtained after dissolving the complex in 0.2% sodium hydroxide solution and subsequent precipitation of the protein as above :*

This experiment was carried out according to the method of Fischer and Hultzsch (3). One g. of the crude substance is treated as above and the precipitate obtained thereby is recombined with iron in the form of ferric chloride in presence of hydrochloric acid at *p*H 2.0. It is then washed with N/2-HCl until free from iron and then dried. Fe, P and N are estimated in the precipitate as usual.

4. *Estimation of* Fe, P *and* N *in the original material :*

Estimations of Fe, P and N in the original materials are carried out exactly in the same manner as described above. Results of analyses are given in Table II.

TABLE II.

*Values of Fe, P and N are given in g. per 100 g. of the
iron-nucleoprotein complex.*

Nature of treatment	Residue.			Filtrate.		
	% Fe	% P	% N	% Fe	% P	% N
1. Treatment with boiling water.	0.0432	1.95	10.1	nil	nil	0.70
2. Treatment with 0.2% NaOH and subsequent precipitation with acetic and trichloroacetic acids at pH 2.0.	nil	1.91	9.8	0.0432	nil	0.90
3. Treatment of the substance as in (2) and then recombining the iron with the residue.	0.0451	1.93	10.2	—	—	—
4. The original substance, without any treatment.	0.0436	1.94	10.8	—	—	—

From the foregoing experiments it is found that on treating the substance with boiling water, only a very small fraction of the total nitrogen is found in the filtrate and this probably represents a portion of the protein nitrogen present as impurity. On treatment with 0.2% sodium hydroxide and subsequent precipitation of the protein with acids, the whole of the iron is found in the acid filtrate with a very small quantity of nitrogen. Then on recombining the residue as obtained in experiment No. 2 with iron, the iron value was found to agree fairly well with that of the original iron-nucleoprotein complex. The fact that the N : P : Fe ratio in the regenerated substance (expt. no. 3) is approximately the same as in the original substance indicates that this is probably the real stoichiometric relationship in the iron-complex and that the substance has been obtained in a fairly pure state.

5. *Estimation of copper :*

Copper was estimated in the original material and was found to be 0.0072%. The whole of it was extracted by trichloroacetic acid.

Separation of nucleic acid from the iron nucleo-protein complex.—The method adopted for the separation and purification of nucleic acid from the iron-nucleoprotein complex is that of Levene and La Forge (4). One g. of the finely powdered substance is mixed with 25 cc. of 0.5% potassium hydroxide solution, allowed to stand overnight and filtered. Then 30 cc.

of saturated aqueous picric acid solution are added to the filtrate, well shaken and filtered through a Buchner funnel. On treatment with 60 cc. of hydrochloric acid (1 part of concentrated acid: 1 part of water), nucleic acid separates from the clear filtrate, which soon adheres to the bottom. The product, which is light yellow because of contamination with picric acid, is suspended in water and brought into solution by the addition of a very slight excess of potassium hydroxide solution. The solution is acidified with acetic acid and poured into ten times its volume of alcohol. The precipitated nucleic acid is washed with absolute alcohol and dried at 95°. The yield is 0.21 g. The nucleic acid thus obtained gave negative tests for protein. The analytical data of the material given below was found to differ to some extent from those of Miescher (5) who worked with fish sperm.

	C	H	N	P
Nuclei acid from iron-nucleo protein (from fish muscle tissue).	35.2%	4.2%	15.1%	8.8%
Miescher's values (from the nucleic acid obtained from fish sperm).	37.8	4.5	15.8	9.7

Mechanism for the liberation of iron from fresh fish tissue by sodium pyrophosphate.—It has been stated in our earlier publication (1) that ferric iron only can form complexes with fish and other animal tissue or egg-yolk. Hence, for the estimation of the ionisable iron, part of which seems to be present in complex combination with nucleoprotein, the iron should first of all be reduced to the ferrous condition. It has been found, however, that sodium pyrophosphate, which is not a reducing agent, can also liberate the iron from egg-yolk and fish muscle tissue. In the case of egg-yolk the liberation of iron is complete within 12 hours but with fish tissue 30 to 40% of the available iron can be accounted for by treating the fish tissue with the above reagent for the same period.

In order to investigate whether the liberation of iron from fish tissue by the action of sodium pyrophosphate may be rendered quantitatively by prolonged extraction, the iron contents of the sodium pyrophosphate soluble and insoluble portions have been separately estimated after stated intervals. Ten g. portions of the finely minced fresh fish muscle tissue are kept with 4% sodium pyrophosphate solution for different periods. The mixture is centrifuged and the residue is washed with pyrophosphate solution and again centrifuged. The iron content, in the residue and in the centrifugate, is estimated by the thiolacetic acid method. The results are given in Table III.

TABLE III.

Iron values are given in mg. per 100 g. of fresh fish tissue.

Periods of contact with sodium pyro-phosphate.	Ionisable iron in the filtrate.	Ionisable iron in the residue.	Total ionisable iron.
24 hours.	0.21 mg.	0.42 mg.	0.63 mg.
48	0.38	0.26	0.64
60	0.52	0.13	0.65
72	0.63	nil	0.64

From the above results, it is found that on increasing the period of treatment with sodium pyrophosphate the iron value in the filtrate increases and that of the residue decreases and after about 72 hours the extraction is complete.

Effect of heating on the liberation of ionisable iron by sodium pyrophosphate.—In order to find out whether heating or boiling has any effect on the liberation of iron by pyrophosphate, the above experiments have been repeated but the mixtures are heated on water-bath for different periods of time and the iron values obtained with the same sample of fish tissue are shown in Table IV.

TABLE IV.

Iron values are given in mg. per 100 g. of fresh fish tissue.

Time of heating in minutes.	Iron in the filtrate.	Iron in the residue.	Total ionisable iron.
10 hours.	0.53 mg.	0.12 mg.	0.65 mg.
20	0.64	nil	0.64
30	0.63	nil	0.63
40	0.65	nil	0.65

The above results indicate that the complete extraction of ionisable iron can be effected by sodium pyrophosphate solution even in 20 minutes, simply by heating the mixture on water-bath.

Tingey (6) and Bruckmann and Zondek (7) have also observed that complete extraction of non-hematin iron with sodium pyrophosphate requires a long time (from 1 to 3 days) which can be shortened by heating.

4

The extraction of tissue materials for 30 minutes at room temperature (32°) with sodium pyrophosphate as was done by Tompsett (8) is therefore likely to be erroneous for the estimation of ionisable iron with this reagent. In the case of egg, of course, as we have stated before, complete extraction of iron is possible within a short time without heating.

The behaviour of the iron-complex of the fish tissue towards pyrophosphate.—Ten g. of the fresh minced fish tissue are taken with 15 cc. of 4% sodium pyrophosphate solution and heated for 20 minutes on the water-bath, after which the pyrophosphate soluble portion is separated by centrifuging. The centrifugate is then acidified with trichloroacetic acid and dialysed for 4 hours in 15 cc. of distilled water. After dialysis the substance left in the dialyser gives positive murexide, xanthoproteic and Molisch tests. The residue after being made free from sodium pyrophosphate by washing gives the test for organically bound phosphorus. The residue gives only a faint reaction for iron. Estimation of the ionisable iron in the dialysate reveals that almost all the iron is present in the dialysate in the form of pyrophosphate.

Estimation of amino and nonamino-nitrogen in the pyrophosphate soluble fraction of fish tissue.—In order to estimate what fractions of the total nitrogen of the pyrophosphate soluble fraction are present as amino- and nonamino-nitrogen, the following experiment has been carried out.

Ten g. of the minced fish tissue are treated with sodium pyrophosphate solution and dialysed as mentioned above. The residue left after dialysis is made up to 100 cc. with distilled water, aliquots are taken and the following estimations are made:

(1) Total nitrogen by Kjeldahl method.

(2) Total amino-nitrogen by formol-titration, after hydrolysis with 20% hydrochloric acid at 150° for 8 hours.

(3) Enzymically liberated amino-nitrogen by formol-titration after peptic hydrolysis for 48 hours at *p*H 2.0.

The mean values for nitrogen thus obtained are expressed in g. per 100 g. of fresh tissue.

By Kjeldahl method.	After acid hydrolysis.	After peptic hydrolysis.
0.43	0.35	0.22% N

Therefore, the percentage of nitrogen which appears to be present as non-amino nitrogen is 0.08 (0.43—0.35), and the percentage of nitrogen which is present in the portion that resists peptic hydrolysis and therefore, may be taken as nucleoprotein nitrogen, is 0.13 (0.35—0.22).

From the above experiments, it appears that iron-nucleoprotein complex of fish tissue is soluble in sodium pyrophosphate solution. Sodium pyrophosphate reverts in solution to orthophosphate, slowly when cold and more rapidly when heated and the sodium hydroxide formed during the process liberates the iron from the complex which is normally soluble even in very dilute alkali (0.2%). The iron thus liberated combines with the unchanged sodium pyrophosphate with the formation of ferric-pyrophosphate.

It has been observed that the purified iron-nucleoprotein complex of fish tissue also behaves similarly, with reference to sodium pyrophosphate, though heating of the complex is necessary to get it into solution. This may be due to possible denaturation of the iron-nucleoprotein complex in the process of isolation.

Summary

1. A method for the isolation of the iron-organic complex from fish muscle tissue has been described ; it involves the peptic hydrolysis of the fish tissue for 24 hours and subsequent washing of the residue with alcohol-ether mixture and then drying in vacuum. It contains, C, H, N, P, Fe and Cu.

2. The determination of the N: P: Fe ratio after different kinds of treatment of the substance indicates that these elements are present in a fairly constant and stoichiometric relation in the iron-complex. These facts also indicate its purity.

3. The iron appears to be present in combination with a nucleo-protein and the corresponding nucleic acid has been separated from it.

4. The substance is insoluble in various organic and inorganic solvents, but is soluble in dilute alkali solutions with the liberation of iron, which can also be released by hydrolysis with moderately strong mineral acids.

5. The whole of the iron in the complex is present in ionisable form and can be estimated quantitatively by the modified method of Hill (2).

6. The copper, present in the complex, is apparently in a loose combination and it can be quantitatively released by simple treatment with 20% trichloroacetic acid.

7. The mechanism for the liberation of iron from fish tissue by sodium pyrophosphate has been studied. Sodium pyrophosphate is transformed in

solution to the orthophosphate, slowly when cold and more rapidly when heated and the sodium hydroxide formed during the process apparently liberates the iron from the complex, as mentioned under 4.

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OBSERVATIONS ON THE STABILITY OF NICOTINIC ACID AND ITS URINARY EXCRETION

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Since the activity of nicotinic acid in the treatment of pellagra and related disturbances in human body (Fouts *et al*, 1) and similar conditions in dogs, pigs and monkeys (Elvehjem *et al*, 2 ; Chick *et al*, 3 ; Harris, 4) had been established, the question of its function in the physiological economy has attracted considerable attention. One direction in which work has proceeded is the study of the urinary excretion of nicotinic acid ; and various colorimetric methods have been proposed for its estimation by different workers, such as von Euler, Schlenk, Heiwinkel and Hogberg (5), Swaminathan (6), Bandier and Hald (7) and Harris and Raymond (8). The principle of all these methods depends upon the estimation of the yellow colour formed by the action of cyanogen bromide in the presence of a primary or secondary aromatic amine, a reaction which was first observed by König (9) with a pyridine derivative.

There are certain points in this method, which require investigation. Despite the fact that nicotinic acid is a relatively stable compound, Melnick and Field (10) state that they have observed a rather rapid decrease in its concentration, when a dilute aqueous solution of nicotinic acid (10 μg per cc.) is allowed to stand at room temperature, and they consider this decrease to be due to bacterial action. No data, however, are given. In connection with the estimation of nicotinic acid, this question, however, is of considerable importance, particularly in the tropics, where the room temperature is higher and therefore more favourable for microbial growth. In the present work, therefore, the stability of nicotinic acid was investigated under varied conditions and subsequently the urinary excretion of nicotinic acid was studied with healthy Bengali subjects.

EXPERIMENTAL

The Stability of Nicotinic Acid in Aqueous Solution.—An aqueous solution of nicotinic acid (1 mg. per cc.) was prepared and 5 cc. of this solution were diluted to 250 cc. with water. Aliquots (20 cc. portions) of the diluted solution were taken in conical flasks of 50 cc. capacity, kept at room temperature (26—28°) and treated as follows:

(1) Diluted to 40 cc. with water.
(2) Ethyl alcohol (20 cc. of 96%) added.
(3) Diluted to 40 cc. with water and adjusted to pH 8.5.
(4) Diluted to 40 cc. with water and adjusted to pH 2.5.
(5) Diluted to 40 cc. with water and adjusted to pH 3.
(6) Diluted to 40 cc. with water and adjusted to pH 4.5.
(7) Diluted to 40 cc. with water and adjusted to pH 5.
(8) Diluted to 40 cc. with water, adjusted to pH 4.5 and
　　4 drops of toluene added.
(9) Treated with 20 mg. of sodium chloride and diluted to 40 cc.
　　with water.
(10) Diluted to 40 cc. with water, adjusted to pH 4.5 and sterilized with
　　steam at 10 lb. pressure for 20 minutes.

The estimations were carried out commencing from the second day after the solutions had been prepared. They were carried out on alternate days for 7 days and finally on the 15th day. The results are given in Table I.

TABLE I.

Solution No.	pH	Taken	2nd day Found	4th day Found	6th day Found	8th day Found	15th day Found
1.	6.2	40*	40	40	40	40	30
2.	5.6 (with alcohol)	40	40	40	40	40	40
3.	8.5	40	40	40	40	40	40
4.	2.5	40	40	40	38	35	10
5.	3	40	30	0	0	0	0
6.	4.5	40	35	0	0	0	0
7.	5	40	36	0	0	0	0
8.	4.5 (with toluene)	40	40	40	40	40	40
9.	6.2 (with NaCl)	40	40	40	40	40	34
10.	4.5 (sterilized)	40	40	40	40	40	40

*values are given in μg

Solution No. 6, after its nicotinic acid content .had disappeared, was inoculated into (*a*) the sterilized solution of nicotinic acid at *p*H 4.5, *i.e.*, solution No. 10, which still contained nicotinic acid and (*b*) into three nutrient peptone media at *p*H 3, 4.5 and 6 respectively. The nicotinic acid content of the sterile solution (No: 10) was found to be *nil* after three days. In the peptone media, bacteria grew within two to three days, the maximum growth being obtained in the medium at *p*H 3.

Urinary Excretion of Nicotinic Acid.—In the present work investigations were carried out on the nicotinic acid excretion of ten healthy Bengalis of whom all were males and eight were adults and two children as a preliminary to the study of the factors concerned in nicotinic acid metabolism and to the determination of the nicotinic acid requirement. The subjects were all non-smokers. Swaminathan's method (11) was used for these estimations and the results described in the preceding section were helpful in preparing stable nicotinic acid standards necessary for comparison. Along with the determination of the nicotinic acid content of urine, known amounts of nicotinic acid were added to the urine samples in order to test how far the method was satisfactory for estimating the added amounts. In all cases, it was observed that 95—100% of the added nicotinic acid could be estimated. We have found that if appreciable quantities of coal gas are present in the atmosphere, the values for nicotinic acid can be considerably vitiated, owing apparently to the presence of traces of pyridine in coal gas. This is a point worthwhile bearing in mind while working in an ordinary laboratory.

The results obtained with the urine of Bengali subjects are given in Table II.

TABLE II

Male subject No.	Age.	24 hours' urine.	Nicotinic acid in 24 hours' urine.
1.	30 years	1325 cc.	5.3 mg.
2.	26	1620	3.8
3.	28	1700	4.4
4.	35	1470	3.5
5.	31	1200	3.0
6.	15	1150	3.5
7.	36	1590	5.3
8.	25	1850	3.3
9.	5	1075	2.7
10.	5	600	1.4

Twenty-four hours' urine was collected and preserved with toluene (free from pyridine) and kept in a refrigerator. It was found that the nicotinic

acid content of the urine remained unaltered for one month. The results with two such samples of urine are given in Table III.

TABLE III.

Figures indicate mg. of nicotinic acid per 50 cc. urine.

Sample No.	1st day.	7th day.	14th day.	21st day.	30th day.
1.	0.20	0.19	0.21	0.20	0.20
2.	0.12	0.13	0.12	0.12	0.12

DISCUSSION AND SUMMARY

The stability of nicotinic acid in dilute aqueous solution under varying conditions has been studied. Such solutions are quickly contaminated with bacteria at room temperature (26—28°) and nicotinic acid disappears, the disappearance being quickest between pH 3 and 5. At pH's, higher or lower, nicotinic acid tends to be stabler. At pH 8.5 it is quite stable. Sodium chloride does not help stability, but alcohol, toluene as well as sterilization confer complete stability.

Values of urinary nicotinic acid excreted by 8 Bengali healthy adult male subjects (non-smokers) during 24 hours were found to vary between 3 and 5.3 mg., while those of two boys of 5 years of age were 2.7 and 1.4 mg. Harris and Raymond (8) have also observed excretion of the order of 3—5 mg. per day, which would seem to indicate that there is not much difference in this respect between the British and Bengali subjects. These figures may be compared with those given by Swaminathan (11), who finds that the average excretion of nicotinic acid by wheat eaters is 6.8 mg. and of rice-eaters 3.2 mg.

My thanks are due to Prof. B. C. Guha for his advice.

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THE ROLE OF VITAMIN C IN INFECTION: EFFECT OF ADMINISTRATION OF ASCORBIC ACID ON THE URINARY EXCRETION OF COMBINED ASCORBIC ACID BY NORMAL AND TUBERCULAR SUBJECTS AND BY GUINEA-PIGS

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Several observers (1—9) have reported that patients suffering from tuberculosis are unsaturated with vitamin C. The rôle of vitamin C in tuberculosis has been studied by Petter (10) and by Sande (11). They have reported that vitamin C combined with gold therapy reduced the tendency to hæmorrhage. Trautwein (4) treated tubercular patients with intravenous injections of vitamin C, 200 mg. per day, until the patients were saturated with vitamin C. He observed remarkable improvement in the condition of his patients as manifested by gain in weight, fall in temperature. decrease in red cell sedimentation rate and a favourable blood picture. Sweany, Clancy, Radford and Hunter (12) studied extensively the rôle of vitamin C in tuberculosis. They tried to estimate the vitamin C content of the body in normal persons and in persons suffering from tuberculosis. The vitamin C content of the normal adult human body varied between 3 and 6 g. In active tuberculosis the total quantity of vitamin C was less than 300 mg. even after a fair intake of vitamin C. In advanced disease, muscle, fatty tissues, bone and skin lost nearly all their reserve of vitamin C while the bulk of the vitamin was retained in tissues like brain, liver, hypophysis, adrenals, gonads, pancreas and spleen. They administered (i) an orange

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flavoured preparation containing vitamin C, (ii) orange juice containing the equivalent amount of vitamin C and (iii) a synthetic orange flavoured preparation without vitamin C to three groups of patients suffering from pulmonary tuberculosis for a period of six months. The death rate in the control group receiving no vitamin C proved to be the highest. The patients in all the three groups who lived after six months of treatment did not, however, show any marked difference in body weight, skiagram, etc. in the different groups. Bauer and Vorwerk (5) are of opinion that the action of vitamin C is not specific but it only improves the general condition. Heise, Martin and Schwartz (2), on the other hand, observed no clinical improvement after treatment with vitamin C in tuberculosis.

It has previously been reported by us (8—9) that patients suffering from acute pulmonary tuberculosis excrete relatively more combined ascorbic acid and less free ascorbic acid in the urine than normal subjects. It was suggested that perhaps the vitamin combines with the toxins or toxic metabolites formed in the system by the tubercle bacilli and tries to eliminate them in the urine as combined ascorbic acid. It was of interest to know what would be the effect of ingestion of ascorbic acid in large doses on the general condition of the tubercular patients and on the excretion of free, dehydro- and combined forms of ascorbic acid in tubercular and normal subjects.

EXPERIMENTAL

Effect of the ingestion of ascorbic acid on the excretion of combined ascorbic acid by tubercular patients.

Fifteen patients suffering from acute pulmonary tuberculosis and nine healthy adults were given 700 mg. ascorbic acid and twenty-four hours' urinary excretion of free, dehydro- and combined forms of ascorbic acid was determined both before and twenty-four hours after the ingestion of ascorbic acid by the method previously described (9).

The results given in Tables I and II indicate that the excretion of combined ascorbic acid is not increased after administration of a single high dose of ascorbic acid.

To note the effect of prolonged administration of ascorbic acid, fifteen patients suffering from acute pulmonary tuberculosis were fed with 700 mg. ascorbic acid per 10 stone body-weight from the second day of the experiment for a period varying between seven and twelve days. All the patients selected had hæmoptysis. Their sputum contained tubercle bacilli and they were getting an evening rise of temperature. The patients received a diet consisting of boiled rice, *dal* (aqueous decoction of pulses), a vegetable curry, fish soup containing 50 g. fish, one pint of milk, chapati made from whole

wheat flour and one lemon per day. During the days of the experiment morning and evening temperatures, rate of the radial pulse and respiration, treatment undertaken, weights, twenty-four hours' urinary excretion of free, dehydro- and combined ascorbic acids etc. were recorded. These are shown in Fig. (1—14) and Table III shows the urinary excretion of free, dehydro- and combined ascorbic acids.

It will be seen from the figures that there is no relation between the rise of temperature and the excretion of ascorbic acid. In most of the cases as the excretion curve of free ascorbic acid rises, the combined ascorbic acid curve comes down. Combined ascorbic acid ceases to be excreted after prolonged administration of ascorbic acid while the excretion of free ascorbic acid is increased.

Effect of the ingestion and injection of ascorbic acid on the excretion of combined ascorbic acid by normal subjects.

Six normal individuals were also fed daily with 70 mg. ascorbic acid per stone body-weight and it was observed that the urinary excretion of combined ascorbic acid gradually disappeared. Results are given in Table IV. In these cases combined ascorbic acid disappeared much more quickly than in the tuberculous patients.

The effect of the intramuscular injection of ascorbic acid on the urinary excretion of combined ascorbic acid has also been studied in five normal individuals. The results are given in Table V.

Effect of the injection of ascorbic acid on the excretion of combined ascorbic acid by guinea-pigs.

In order to compare the behaviour of human subjects with that of guinea-pigs, twenty-four hours' urinary excretion of free, dehydro- and combined ascorbic acid was also determined with guinea-pigs kept under controlled conditions. Three groups of guinea-pigs of weights varying between 450 g. and 550 g. each containing twelve animals were placed in different cages. One group was given a scorbutic diet for three weeks, the second group received normal diet consisting of green grass and germinated gram for three weeks, while the third group which was also on a normal diet received a daily intraperitoneal injection of 100 mg. ascorbic acid for two weeks. The animals were then placed in metabolism cages in batches of three and their combined urine was collected for twenty-four hours over 2 cc. of 50 per cent. sulphuric acid. The amounts of free, dehydro- and combined ascorbic acids in urine were determined. The results are given in Tables VI—VIII. Combined ascorbic acid which is present in urine of scorbutic and normal guinea-pigs was found to be absent in the urine of guinea-pigs receiving injections of ascorbic acid.
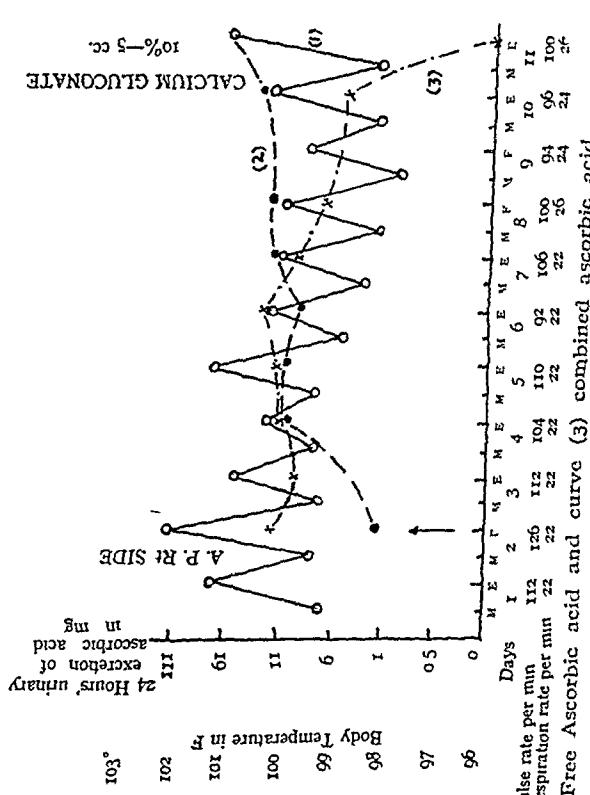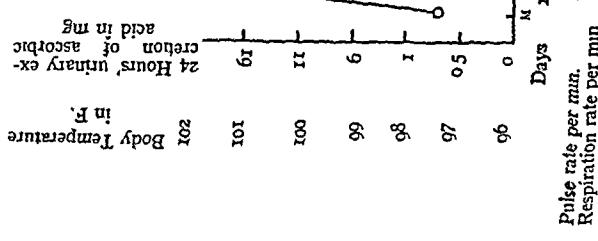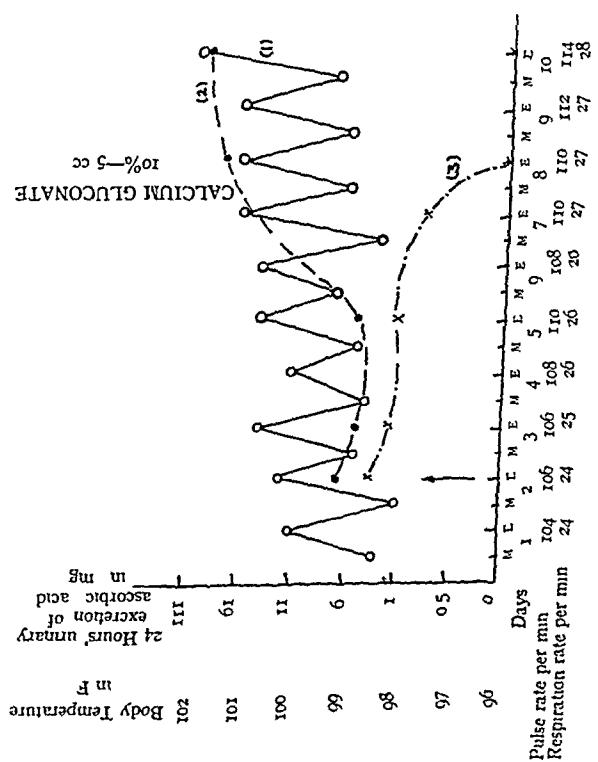
M = Morning E = Evening curve (1) Temperature curve (2) Free Ascorbic acid and curve (3) combined ascorbic acid
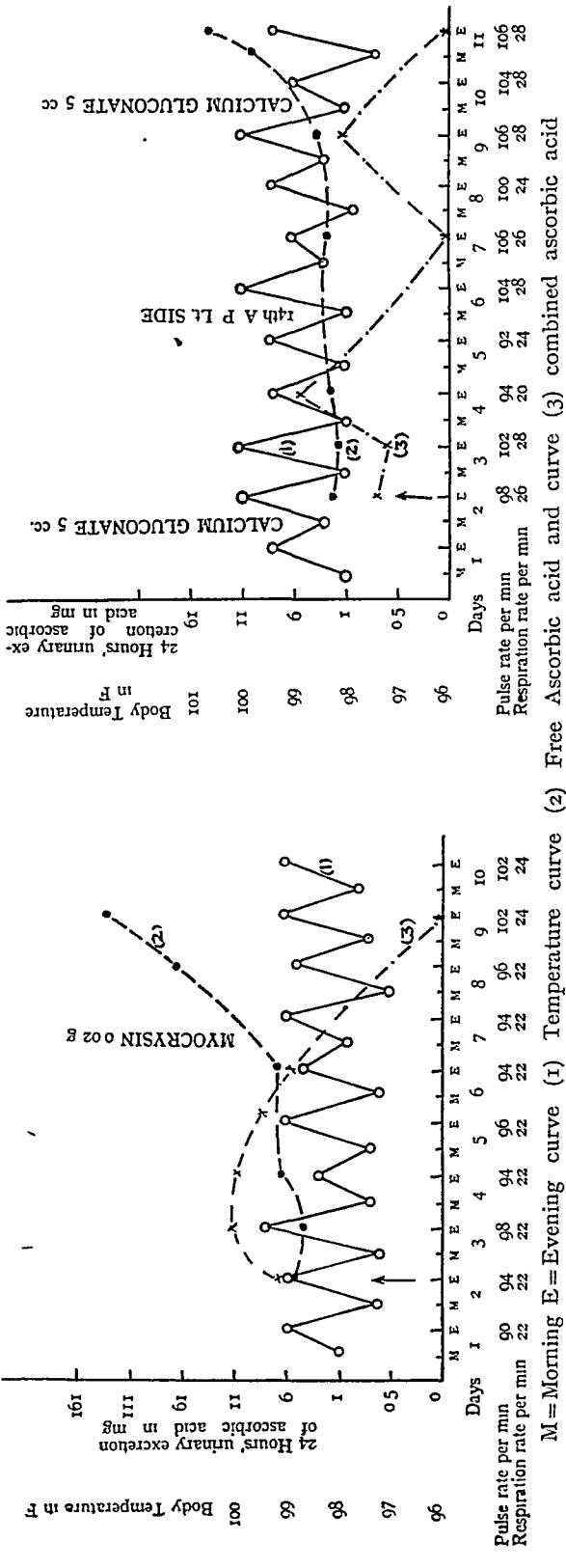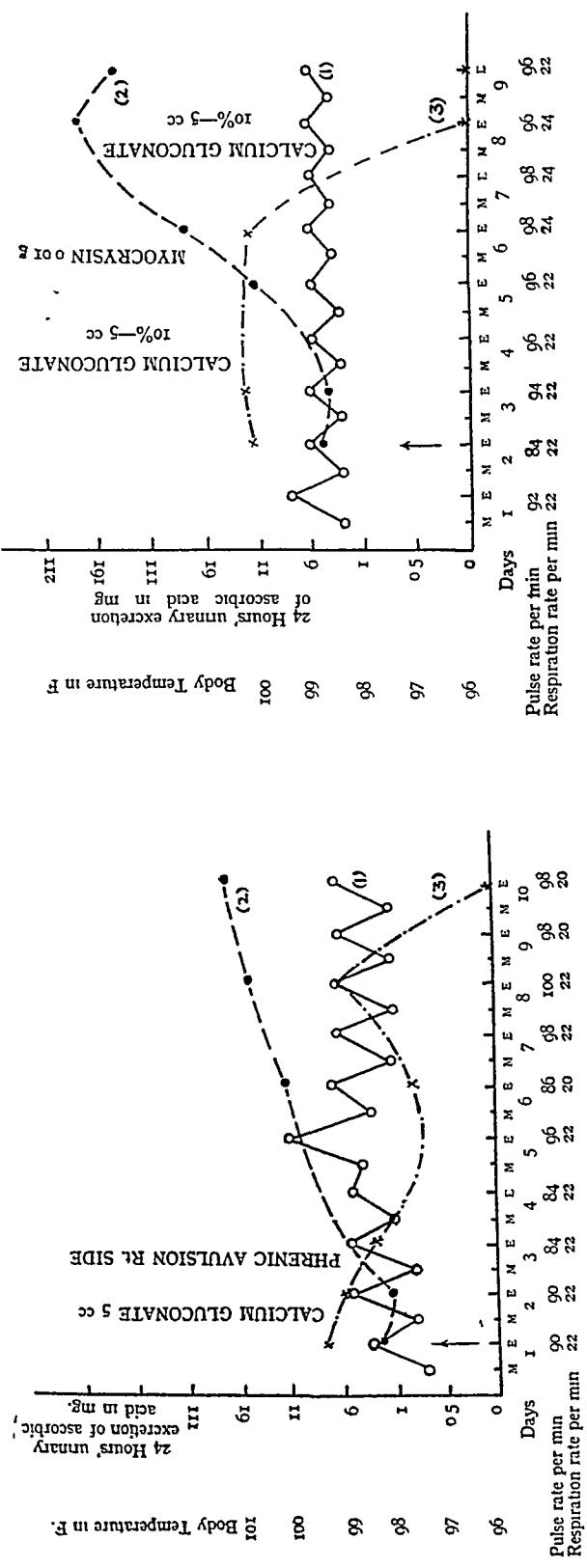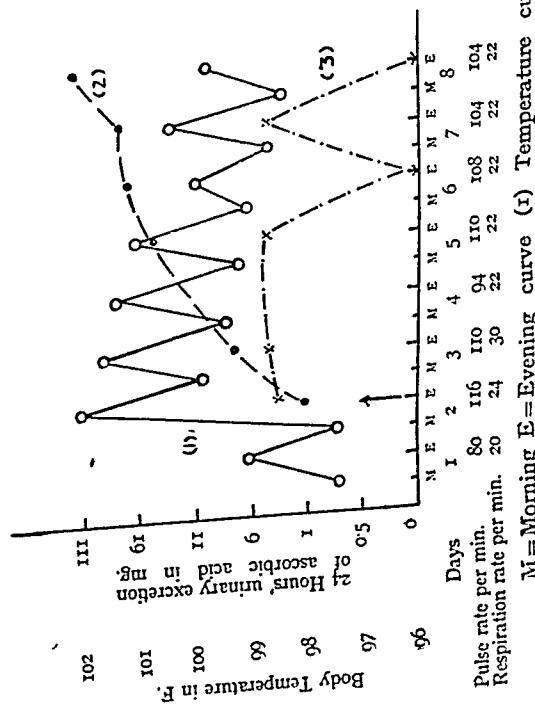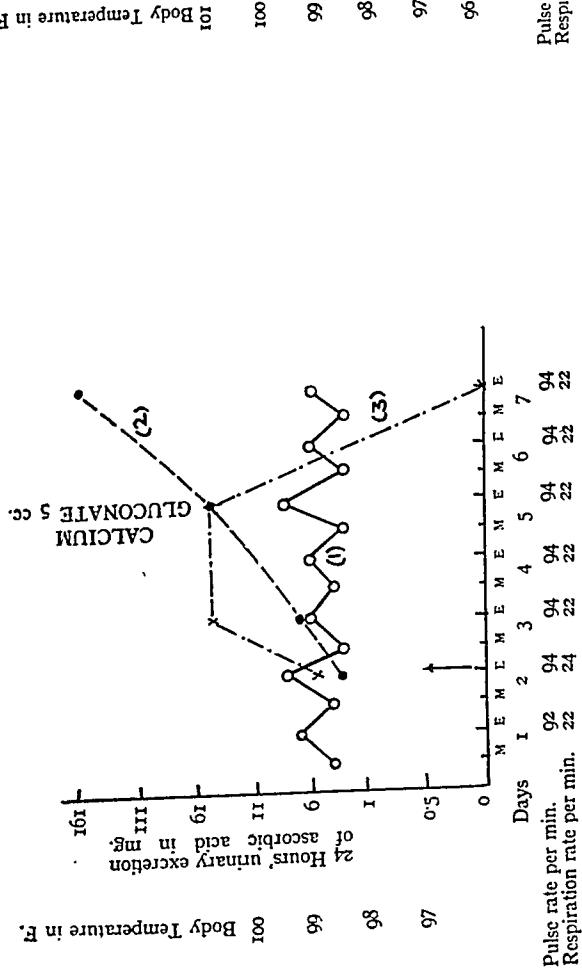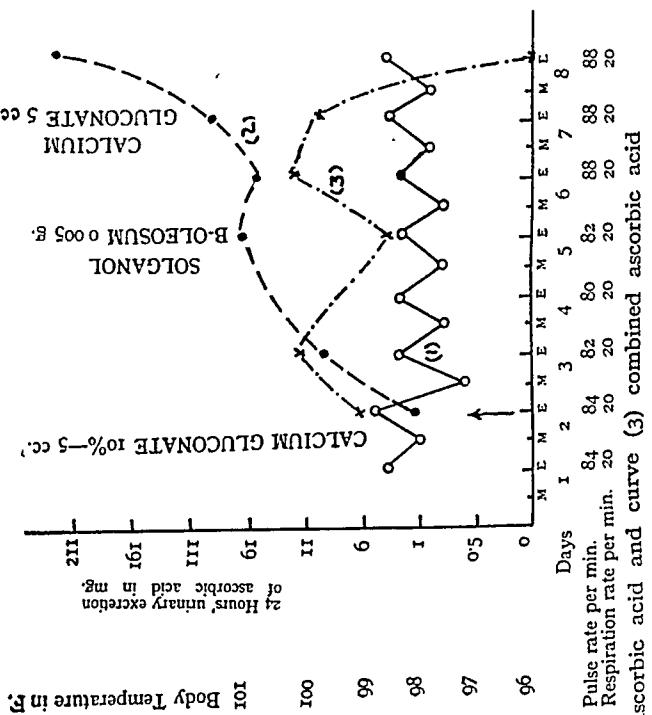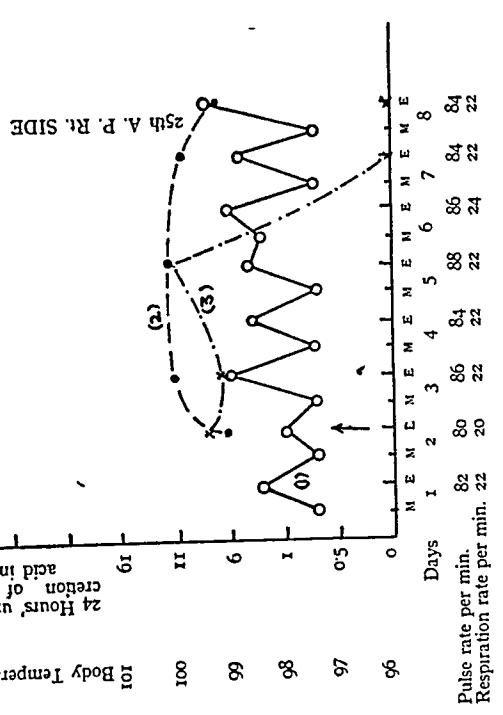
M = Morning   E = Evening   curve (1) Temperature curve   (2) Free Ascorbic acid and curve (3) combined ascorbic acid

M=Morning E=Evening curve (1) Temperature curve (2) Free
Ascorbic acid and curve (3) combined ascorbic acid
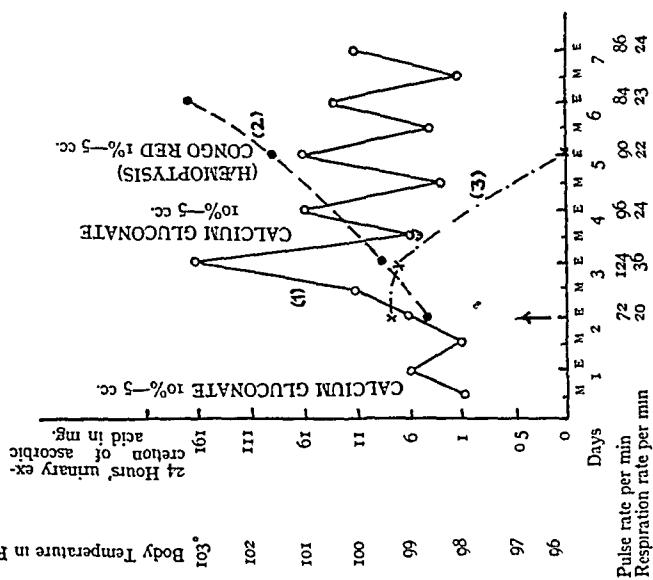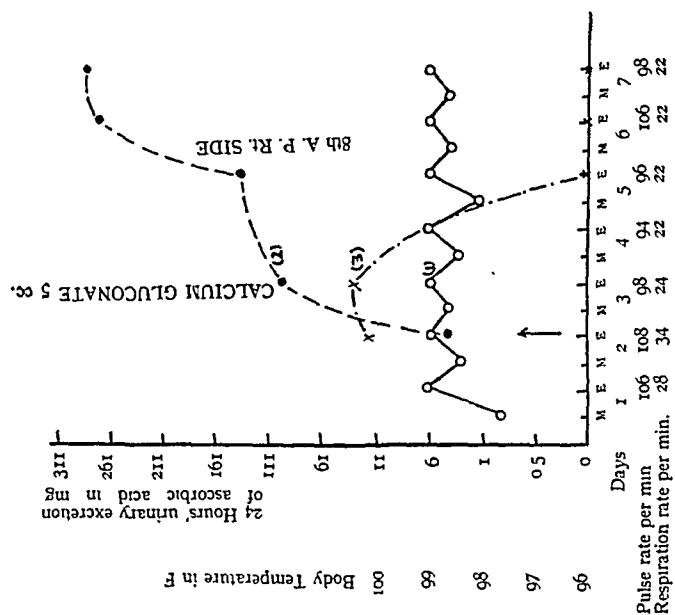
M=Morning E=Evening curve (1) Temperature curve (2) Free Ascorbic acid and curve (3) combined ascorbic acid

TABLE I.

24 hours' urinary excretion of ascorbic acid in mg. in cases suffering from acute pulmonary tuberculosis.

Patient No.	Name	Age	Before ingestion of ascorbic acid.				After ingestion of ascorbic acid.			
			Free.	Dehydro.	Combined.	Total.	Free.	Dehydro.	Combined.	Total.
1.	R. B. K.	28	1.09	4.91	18.33	24.33	11.25	12.92	22.55	46.72[1]
2.	J. N. G.	32	1.06	0.23	10.05	11.34	1.65	0	21.75	23.40[2]
3.	A. C. B.	20	2.04	6.70	8.51	17.25	4.46	5.66	2.60	12.72[3]
4.	H. A. R.	30	3.04	0	12.74	15.78	2.15	2.62	0.77	5.54[4]
5.	D. M. C.	24	2.71	6.08	8.96	17.75	2.32	0.18	6.99	9.49[5]
6.	P. S.	29	2.74	9.83	1.79	14.36	3.50	3.57	7.84	14.91[6]
7.	T. C. P.	24	3.66	0	1.66	5.32	5.56	6.96	9.73	22.25[7]
8.	S. K. S.	28	3.25	1.65	11.94	16.84	48.21	15.01	4.62	67.84[8]
9.	R. U.	36	3.43	2.42	14.47	20.32	5.11	1.18	9.90	16.19[9]
10.	B. K. M.	28	5.42	5.27	24.37	35.06	7.95	0.19	12.46	20.60[10]
11.	S. N. D.	22	3.78	4.96	10.39	19.13	12.11	4.02	5.72	21.85[11]
12.	N. C. S.	32	9.02	1.06	19.78	29.86	4.28	1.90	0.43	6.61[12]
13.	K. C. B.	26	8.00	4.48	33.35	45.83	34.11	5.80	17.67	57.58[13]
14.	R.	29	6.18	2.66	7.23	16.07	20.30	2.65	15.41	37.36[14]
15.	A. C. B.	14	9.90	6.96	14.03	30.9	6.96	6.96	0.58	14.50[15]

1. Left sided affection. Evening temperature 99°.5F.
2. Left sided affection. Temperature 99°.4—101°F. Very acute case.
3. Left sided affection. Temperature 98°.4—100°F.
4. Weight decreasing. Diabetic. Both sided affection. Evening temperature 99°F.
5. Left sided affection. Temperature 97°.4—100°F.
6. Both sided affection. Evening temperature 98°.4. Quescent case.
7. Right sided affection. Very acute case.
8. Both sided affection. Evening temperature 99°F.
9. Both sided affection. Temperature 102°F. Very acute case.
10. Both sided affection. Temperature 102°F. Very acute case.
11. Both sided affection. Temperature 99°—100°F. Very acute case.
12. Both sided affection. Temperature 99°—100°F. Very acute case.
13. Right sided affection. No rise of temperature.
14. Right sided affection. No rise of temperature.
15. Right sided affection. No rise of temperature.

TABLE II.

24 hours' urinary excretion of ascorbic acid in mg. in normal individuals.

Serial No.	Name.	Age in years.	Before ingestion of ascorbic acid.				After ingestion of ascorbic acid.			
			Free.	Dehydro.	Combined	Total.	Free.	Dehydro.	Combined	Total.
1.	N. C.	21	31.70	5.45	22.40	59.55	64.24	18.89	41.81	124.94
2.	G. B.	27	26.60	1.79	11.29	39.68	68.45	2.54	4.07	75.06
3.	S. N.	21	28.69	0	11.58	40.27	93.02	0	23.89	116.91
4.	N. B.	23	48.37	25.50	27.80	101.67	85.92	0	41.31	127.23
5.	D. B.	28	38.90	15.28	32.82	87.00	79.69	10.34	30.38	120.41
6.	I. M.	36	67.11	14.11	3.22	84.44	71.75	9.35	49.33	130.43
7.	B. B.	38	29.43	0	7.39	36.82	60.79	0	26.11	86.90
8.	G. B.	27	71.42	4.53	19.77	95.72	209.70	28.71	12.15	250.56
9.	C. B.	30	58.39	0	8.15	66.54	80.08	8.75	13.04	101.87

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TABLE III.

24 hours' urinary excretion of ascorbic acid in mg. in patients suffering from acute pulmonary tuberculosis. The patients were given ascorbic acid (70 mg. per stone body weight) daily after collecting first 24 hours' urine.

No.	Patient.	Age.	Ascorbic acid.	Days.						
				1	2	3	4	6	8	10
1.	K. P. P.	17	Free	10.00	2.24	5.62		17.78	35.33	56.39
			Dehydro	0.43	0.44	0		0	1.50	0
			Combined	1.65	1.89	6.04		2.80	0	0
2.	N. U.	28	Free	2.36	1.77	2.51		2.71	3.77	40.90
			Dehydro	0	0.15	0.56		0	0	2.88
			Combined	0.72	0.64	5.25		0	1.24	0
3.	D. C. S.	22	Free	6.39	12.40		12.90	10.94	7.37	
			Dehydro	1.48	3.93		1.93	0	0	
			Combined	8.03	6.79		12.55	0	0	
4.	R. M.	26	Free	3.07	7.00		48.04	157.27		
			Dehydro	0	26.83		54.03	42.23		
			Combined	8.48	47.45		49.13	0		
5.	N. N. G.	25	Free	6.81	4.86		5.00	50.89		
			Dehydro	1.32	1.48		1.77	28.10		
			Combined	3.59	5.26		1.30	0		
6.	B. N. D.	20	Free	4.31	8.89		86.69	165.15		
			Dehydro	3.80	1.73		0	0		
			Combined	7.71	7.38		0	0		

TABLE III.—(Contd.)

24 hours' urinary excretion of ascorbic acid in mg. in patients suffering from acute pulmonary tuberculosis. The patients were given ascorbic acid (70 mg. per stone body weight) daily after collecting first 24 hours' urine.

No.	Patient.	Age.	Ascorbic acid.	1	2	4	5	6	7	8	9	10
7.	O. H.	36	Free	4.17	6.35	6.28		65.95	133.24	80.76		
			Dehydro	3.22	1.87	0.72		0	0	11.07		
			Combined	12.49	10.43	13.01		10.99	0	0		
8.	D. M. C.	25	Free	7.39		24.67		35.13	9.00	15.18		
			Dehydro	2.63				2.53	3.60	3.51		
			Combined	1.23		9.37		8.34	5.47	0		
9.	T. C. C.	31	Free	4.18	96.25	33.84	270.83	277.88				
			Dehydro	5.10	34.49	31.39	0	0				
			Combined	14.47	30.40	0	0	0				
10.	B. C. G. N.	30	Free	1.01	7.33	47.09	69.27	73.14	117.30			
			Dehydro	0	3.31	0	19.19	0	25.78			
			Combined	3.07	4.36	4.60	0	4.81	0			
11.	A. P.	18	Free	1.54	9.70	69.50	52.28	89.21	226.17			
			Dehydro	0	2.70	0	5.83	0	0			
			Combined	6.16	19.29	3.57	22.13	10.65	0			
12.	S. N. P.	38	Free	14.53	266.67	49.52	315.38	222.47				
			Dehydro	0	0	0	0	0				
			Combined	19.30	44.51	21.36	0	0				
13.	P. S.	30	Free	1.84	4.29	10.40	10.66	9.46	12.95		27.95	65.61
			Dehydro	0.52	0	0	0.51	0	0.82		22.17	0
			Combined	19.25	9.71	15.36	15.85	37.12	9.78		5.49	0
14.	S. N. D.	30	Free	5.25	5.08	6.77	3.77		16.11	104.73	16.78	120.28
			Dehydro	0.28	6.80	0.73	2.79		5.54		0	0
			Combined	37.97	12.25	10.50	15.27		12.62		0	0
15.	T. P. H.	32	Free	5.25	4.68		77.37		175.91		143.00	
			Dehydro	0.86	0.33		11.92		0		0	
			Combined	12.51	25.81		16.33		0		0	

Days.

TABLE IV.

Excretion of ascorbic acid in 24 hours' output of urine by six normal healthy individuals. The individuals were given per month 700 mg. ascorbic acid per 10 stone body weight from the second day after collection of 24 hours' urine. The figures are expressed in milligram of free ascorbic acid.

No.	Name.	Age.	Ascorbic acid.	Days.				
				2	3	4	5	6
1.	S. B.	30	Free	30.66	490.00	378.8		
			Dehydro	3.46	0	0		
			Combined	6.51	0	0		
2.	R. P.	30	Free	3.42	13.29	170.47	241.64	110.75
			Dehydro	0	11.19	23.53	0	0
			Combined	6.12	5.18	0	0	0
3.	S. R.	36	Free	8.68	176.36	215.82	259.79	
			Dehydro	2.65	30.85	0	0	
			Combined	9.27	0	0	0	
4.	S. M.	36	Free	9.60	17.10	299.69	195.56	
			Dehydro	0	0	0	5.36	
			Combined	0.86	16.06	0	0	
5.	N. M.	20	Free	5.59	9.96	19.55	118.36	
			Dehydro	2.97	1.85	0	0	
			Combined	1.85	7.25	9.92	0	
6.	B. D.	26	Free	17.07	136.17	198.10		278.55
			Dehydro	0	0	0		0
			Combined	16.32	15.42	0		0

TABLE V.

24 hours' urinary excretion of ascorbic acid expressed in milligram of free ascorbic acid in five healthy individuals. They were given daily intramuscular injection of ascorbic acid, 70 mg. per stone body weight from the second day after collection of 24 hours' urine.

No.	Name.	Age.	Ascorbic acid.	Days.				
				2	3	4	5	6
1.	B. D.	26	Free	9.22	334.5	617.49		
			Dehydro	3.43	22.3	o		
			Combined	7.18	o	o		
2.	S. M.	26	Free	7.60	285.91	190.54		
			Dehydro	o	29.09	o		
			Combined	2.39	o	o		
3.	R. P.	30	Free	4.58	11.30	121.00		
			Dehydro	2.32	9.04	22.38		
			Combined	21.63	21.52	o		
4.	S. R.	36	Free	7.50	7.14	372.5		
			Dehydro	0.71	4.66	o		
			Combined	4.29	5.34	o		
5.	N. M.	20	Free	5.42	28.07	89.52	158.00	269.24
			Dehydro	o	12.18	15.89	o	o
			Combined	0.68	2.78	6.58	o	o

TABLE VI.

24 hours' urinary excretion of ascorbic acid in mg. in guinea-pigs on scorbutic diet.

No.	Batch of 3 guineapigs.	Days.	Free.	Dehydro.	Combined.	Total.	Ratio of combined to free ascorbic acid.
1.	1st	1st	0.12	0.07	0.08	0.27	
		2nd	0.14	0.08	0.21	0.43	
		3rd	0.16	0	0.34	0.50	
	M e a n excretion per animal per day.		0.04	0.02	0.07	0.13	1.75
2.	2nd	1st	0.26	0.14	0.34	0.74	
		2nd	0.22	0.14	0.34	0.71	
		3rd	0.16	0.06	0.24	0.46	
	M e a n excretion per animal per day.		0.07	0.04	0.10	0.21	1.43
3.	3rd	1st	0.03	0.22	0.05	0.30	
		2nd	0.17	0.16	0.22	0.55	
		3rd	0.19	0.07	0.21	0.47	
	M e a n excretion per animal per day.		0.04	0.05	0.05	0.14	1.25
4.	4th	1st	0.40	0.24	0.98	1.62	
		2nd	0.08	0.15	0.11	0.34	
		3rd	0.03	0.03	0.05	0.11	
	M e a n excretion per animal per day.		0.06	0.05	0.13	0.24	2.17

TABLE VII.

24 hours' urinary excretion of ascorbic acid in mg. in groups of three guinea-pigs kept on an ordinary diet consisting of green grass and germinated gram.

No.	Batch of 3 guineapigs.	Days.	Free.	Dehydro.	Combined.	Total.	Ratio of combined to free ascorbic acid.
1.	1st	1st	1.57	0	0.88	2.45	
		2nd	1.56	0.09	2.09	3.74	
		3rd	1.09	0.45	0.58	2.12	
	M e a n excretion per animal per day.		0.47	0.06	0.39	0.92	0.83
2.	2nd	1st	1.39	0	0.78	2.17	
		2nd	2.00	0	0.67	2.67	
	M e a n excretion per animal per day.		0.57	0	0.24	0.81	0.42

TABLE VII (*Contd.*)

24 hours' urinary excretion of ascorbic acid in mg. in groups of three guinea-pigs kept on an ordinary diet consisting of green grass and germinated gram.

No.	Batch of 3 guineapigs.	Days.	Free.	Dehydro.	Combined.	Total.	Ratio of combined to free ascorbic acid.
3.	3rd	1st	1.28	0	0.88	2.16	
		2nd	1.31	0.09	0.37	1.77	
		3rd	2.13	0.31	0.13	2.57	
	Mean excretion per animal per day.		0.52	0.04	0.15	0.72	0.19
4.	4th	1st	2.34	0.28	1.03	3.65	
		2nd	2.11	0	0.89	3.00	
		3rd	2.49	0.35	0.37	3.21	
	Mean excretion per animal per day.		0.77	0.07	0.25	1.09	0.32

TABLE VIII.

24 hours' urinary excretion of ascorbic acid in mg. in groups of three guinea-pigs on normal diets receiving a daily injection of 100 mg. ascorbic acid for two weeks.

No.	Batch of 3 guineapigs.	Days.	Free.	Dehydro.	Combined.	Total.
1.	1st	1st	123.5	0	0	123.5
		2nd	220.4	0	0	220.4
		3rd	98.0	0	0	98.0
	Mean excretion per animal per day.		49.1	0	0	49.1
2.	2nd	1st	63.72	23.02	0	86.74
		2nd	44.98	7.41	0	52.39
		3rd	78.18	0	0	78.18
	Mean excretion per animal per day.		20.76	3.38	0	24.14
3.	3rd	1st	132.43	4.95	0	137.38
		2nd	58.61	0	0	58.61
		3rd	86.88	0	0	86.88
	Mean excretion per animal per day.		30.88	0.55	0	31.43
4.	4th	1st	123.50	0	0	123.50
		2nd	109.29	0	0	109.29
		3rd	78.18	0	0	78.18
	Mean excretion per animal per day.		34.55	0	0	34.55

DISCUSSION

From the experiments on tuberculous patients, normal human subjects and on guinea-pigs under different controlled conditions it is observed that combined ascorbic acid disappears from the urine after the administration of fairly heavy doses of ascorbic acid for a prolonged period.

In the case of tubercular patients the time taken for disappearance of ascorbic acid is much more longer (4—10 days). In tubercular patients after administration of high doses of ascorbic acid the excretion of free ascorbic acid gradually rises but the value is quite small when compared with the excretion of free ascorbic acid in normal individuals. There was, however, no marked improvement in their clinical condition during the short period varying from six to ten days in which vitamin C was administered. That, however, the rise of ascorbic acid content of the urine is much more slow in tuberculous patients than in normal individuals would tend to show that the ingested vitamin was being needed and utilized by the patients. Administration of vitamin C to tuberculous patients is therefore likely to be beneficial.

The striking point that arises from this investigation is the disappearance of combined ascorbic acid from the urine of normal and tubercular persons as well as of guinea-pigs on continued administration of large doses of ascorbic acid. We had previously thought it possible that combined ascorbic acid was a means of directly eliminating the toxic metabolites of the body. If this were so, it is difficult to see why in tuberculous patients, continued administration of vitamin C would lead to the disappearance of the combined ascorbic acid of urine. A possible explanation may be that in the absence of ascorbic acid the normal metabolism of the food-stuffs and of tissue-constituents may be disturbed and the intermediary products may accumulate, which may be excreted in combination with ascorbic acid in urine. When large doses of ascorbic acid are fed, the metabolism is complete and intermediary products do not accumulate and therefore the excretion of ascorbic acid in combination with these products would tend to diminish. This may happen both in normal and infected conditions, and the quicker disappearance of combined ascorbic acid from urine of normal persons by feeding ascorbic acid may be related to the possibly greater speed in the action of vitamin C in normal individuals. That the ratio of combined ascorbic acid to free ascorbic acid excreted by guinea-pigs is greatly increased in scorbutic condition is shown in Tables VI-VII of this paper. Ascorbic acid would thus act as a detoxicating agent but in an indirect manner.

· If this theory is correct it would also suggest a means of determining the vitamin C status of the body. The minimum dose of vitamin C that

makes combined ascorbic acid disappear from the urine may represent the optimum intake. This, however, may not necessarily represent the dose required to saturate the body, as in experiments with guinea-pigs we have found that the minimum decolorisation time in the intradermal test, representing the saturation of the skin with vitamin C, is reached some time after the combined ascorbic acid has disappeared from the urine on continued feeding of fairly heavy doses of ascorbic acid.

The above theory appears to receive some support from the recent findings of Sealock and Silberstein (13). They have observed that in scorbutic guinea-pigs ingested tyrosine does not undergo complete metabolism and that homogentistic acid, *p*-hydroxyphenyl lactic acid, and *p*-hydroxyphenylpyruvic acid appear in the urine, which disappear on the administration of vitamin C. It was further observed that *l*-ascorbic acid could not be quantitatively replaced in this regard by *d-iso*ascorbic acid and the effects of the two substances were in accord with their actual anti-scorbutic potencies. There is, however, no evidence in their paper that any intermediary products of metabolism were excreted in combined form. Somewhat similar findings have been reported by Levine, Gordon and Marples (14) working with premature infants. They observed that the infants, when fed with vitamin C-free milk, excreted *p*-hydroxyphenyl lactic acid and *p*-hydroxyphenylpyruvic acid, which disappeared on the administration of ascorbic acid and they consider the action of ascorbic acid as specific. Levine, Marples and Gordon (15) observed the same imperfect metabolism of tyrosine when fed to premature infants without the addition of ascorbic acid, but they pointed out that tryptophane did not give rise to its typical intermediary degradation products under the same conditions.

Work is now proceeding on the nature of combined ascorbic acid in urine excreted under different conditions, as such studies are likely to throw further light on the adequacy or otherwise of the interpretation mentioned above which must be regarded as entirely tentative.

SUMMARY

1. The urinary excretion of combined ascorbic acid is not appreciably affected by the administration of a single high dose of ascorbic acid to tuberculous patients.

2. Combined ascorbic acid disappears from the urine of normal human subjects 1—3 days after continued ingestion or injection of ascorbic acid in doses of 70 mg. per stone body-weight, and the excretion of free ascorbic acid is greatly and quickly increased.

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3. Combined ascorbic acid disappears from the urine of tubercular patients more slowly, 4 to 10 days after continued oral administration of ascorbic acid in doses of 70 mg. per stone body-weight, while the excretion of free ascorbic acid is increased also more slowly than in normal subjects. Clinical improvement, in the cases studied, has not been observed within the short period during which ascorbic acid was administered, though the slow increase of free ascorbic acid in the urine would indicate that the ascorbic acid administered was being needed and utilized by the body.

4. Combined ascorbic acid which is present in the urine of scorbutic and normal guinea-pigs is found to be absent in the urine of guinea-pigs receiving injections of high doses of ascorbic acid. The ratio of combined ascorbic acid to free ascorbic acid excreted by guinea-pigs is greatly increased in scorbutic condition.

5. The significance of the disappearance of combined ascorbic acid from the urine on the administration of high doses of ascorbic acid is discussed.

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A RAPID TECHNIQUE FOR THE PRODUCTION OF NUTRITIONAL ANÆMIA IN RATS

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Up till now, various methods have been proposed and adopted for the production of nutritional anæmia in rats. As the biological availability of iron in foodstuffs is determined either with reference to the rate of hæmoglobin regeneration in anæmic rats or to the total absorption of iron under anæmic conditions, a rapid and reliable method for the production of deficiency anæmia with regard to iron seems to be urgently needed. Waddell, Steenbock, Elvehjem and Hart (1) have reported that if newly weaned rats are kept on milk diet for 4—6 weeks, severe anæmia develops. But in our present investigation, we could not find any distinct sign of nutritional anæmia in rats even when their diets were restricted to cow's whole milk for 8 weeks. The hæmoglobin level came down to 71 per cent. only and the total body-iron fell slightly below the normal value (4.1 mg. per 100 g. of body-weight).

The cardiac bleeding method as used by Hahn and Whipple (2), Williamson (3), McCay (4) and others was equally ineffective. Due to the smaller sizes of the animals, the heart is sometimes damaged by repeated puncturing producing thereby unknown pathological conditions, resulting in death during the course of experiment.

The technique, described by Elvehjem and Kemmerer (5) though much more effective, requires much longer time and careful manipulation. Furthrmore, the authors did not take into consideration the total reserve of iron of the animals, which seems to be essential in connection with the determination of biological availability of iron. Taking the decreased blood-hæmoglobin level alone as the criterion for nutritional anæmia, it cannot be definitely said that the animals have been well depleted of the reserve of iron. When the animals are kept on milk diet for a longer time, the reduced hæmoglobin level may arise out of the deficiency of some other factor in which the whole milk may be deficient. Drabkin and Miller (6)

believed that the anæmia produced by milk-feeding was not due to the deficiency of iron but to the deficiency of the pyrrol nuclei necessary for the formation of the porphyrin moiety of hæmoglobin. In our previous publication (7) we have shown that addition of iron alone to the whole milk diet does not help in the regeneration of blood hæmoglobin but increases the total reserve of iron to a great extent. If, on the other hand, nucleic acid is supplemented with the same iron level, the stored iron is utilized for supernormal hæmoglobin formation and the reserve of body iron corresponds almost to the normal value.

So, when the biological availability of iron in a particular foodstuffs is to be studied, it should be preferably done both with regard to the regeneration of blood-hæmoglobin and to the mobilization of the iron reserve in the body.

In our previous publication (7) we have further shown that on supplementing the milk diet of rats with nucleic acid alone, stimulation of hæmopoiesis results upto the second week, after which the blood hæmoglobin and erythrocyte levels fall to a low value. The stored iron of animals is greatly decreased. Hence, we thought it worthwhile investigating whether by allowing the animals to be depleted of their reserve iron by oral administration of nucleic acid, a technique for the production of nutritional anæmia in rats in a quick and reliable manner could be developed.

EXPERIMENTAL

For our present investigation 24 adult rats were taken having comparable weights (45—52 g.). The animals were kept in individual aluminium cages with screened bottoms, specially made for the purpose, and were fed with whole cow's milk as the basal diet. Prior to the beginning of the experiment, the normal blood hæmoglobin and erythrocyte determination were made according to the method previously described (7). As it has been stated by Miller (8) and Rene Loieq (9) that hyper-leucocytosis occurs on the intravenous injection of sodium nucleinate in mice and rats, the total leucocytes determination has also been carried out in the present investigation in order to see whether this hyperleucocytosis is produced which might have some implication in this work. After the determination of hæmoglobin, erythrocytes and leucocytes, 20 animals were fed with a supplement of 5.0 mg. of nucleic acid as its sodium salt per animal per day moistened with two drops of codliver oil, in order to increase the palatability of the supplment. The animals, receiving the supplement, were bled weekly by clipping off the tip of the tail in order to facilitate the formation of more hæmoglobin at the expense of the stored iron by shifting the equilibrium between blood formation and destruction. The remaining animals served as controls on milk diet alone and were not bled. At the end of the fourth week, the addition of supplement was stopped and the animals were kept on milk diet for another 15 days in order to deplete them of any unabsorbed

nucleic acid, after which, the blood hæmoglobin, erythrocyte and leucocyte levels were again determined (Table I). Then after the expiry of the sixth week, the bodies of the animals of the control group on milk diet alone and those of four of the animals receiving supplement were burnt to white ash and the total body iron was determined by the method described earlier (7). The values obtained are given in Table II. The rest of the animals were divided into two groups, having 8 in each group. The diet of the first group was supplemented with 5.0 mg. of nucleic acid and 0.3 mg. of iron as ferric chloride and that of the second group with 0.3 mg. of iron alone, per animal per day. These supplements were continued for four weeks and the hæmoglobin, erythrocyte and leucocyte concentrations were determined weekly as usual. The results are shown in Figs. I, II and III. Each graph represents the average of 8 animals.

TABLE I

Initial and final blood hæmoglobin, erythrocyte and leucocyte levels of rats made anæmic by nucleic acid administration and of those kept on milk diet alone.

Initial.			Final.			Supplement.
Hb. (in %).	R.B.C. (in millions).	W.B.C. (in thousands).	Hb. (in %).	R.B.C. (in millions).	W.B.C. (in thousands).	5 mg. of nucleic acid as sodium salt per animal per day.
96.0	7.4	7.2	43.0	3.2	6.8	
89.2	8.2	6.3	46.2	3.3	6.2	
92.0	8.1	6.9	38.3	4.0	7.1	
98.0	7.9	6.8	40.2	2.8	6.9	
100	8.6	7.3	41.6	2.6	7.1	
96.0	7.6	6.6	45.3	3.0	6.3	
95.5	6.8	7.3	36.2	3.6	7.5	
97.5	7.2	7.0	51.3	4.3	6.8	
93.2	7.8	6.0	46.0	4.2	6.3	
86.8	8.1	6.5	42.0	4.0	6.4	
90.3	8.0	7.1	49.3	3.9	7.6	
88.5	8.1	7.3	36.2	3.6	7.0	
87.5	7.2	6.7	39.1	4.6	6.9	
93.0	8.3	7.5	38.0	3.2	7.9	
95.0	8.6	6.8	43.1	3.0	6.7	
87.0	6.9	6.2	46.0	3.8	6.3	
82.3	6.6	6.0	48.0	4.2	6.4	
88.0	7.2	7.3	40.0	4.0	7.0	
86.0	6.9	6.5	38.9	4.2	6.8	
90.8	8.2	7.2	43.0	3.8	7.1	
98.2	7.8	7.6	76.0	4.8	7.5	No supplement.
100	8.5	6.9	81.0	5.2	7.3	
105	8.8	7.2	69.5	5.1	7.2	
97.0	7.9	6.7	68.0	5.3	5.3	

TABLE II

Iron content of the animal body, with and without nucleic acid supplement, expressed in mg. per 100 g. body-weight.

| Iron content.	Supplement.
1.8	50 mg. of nucleic acid as sodium
1.6	salt per animal per day.
2.1	
2.3	
3.6	No supplement.
3.2	
2.9	
3.1	
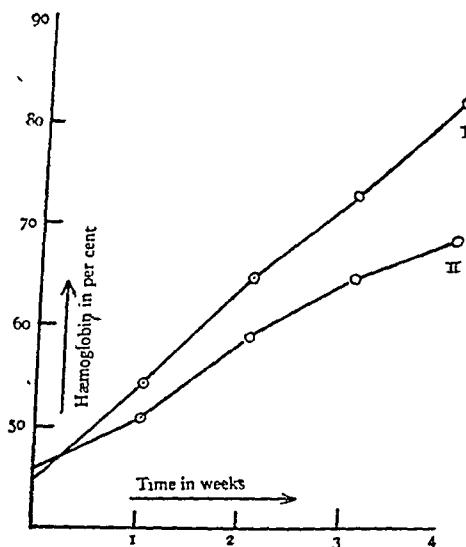
FIG. 1

I. Represents the increase in Hb% of the animals receiving 5.0 mg. of nucleic acid *plus* 0.3 mg. of iron.

II. Represents the increase in Hb% of the animals receiving 0.3 mg. or iron only.

FIG. 2
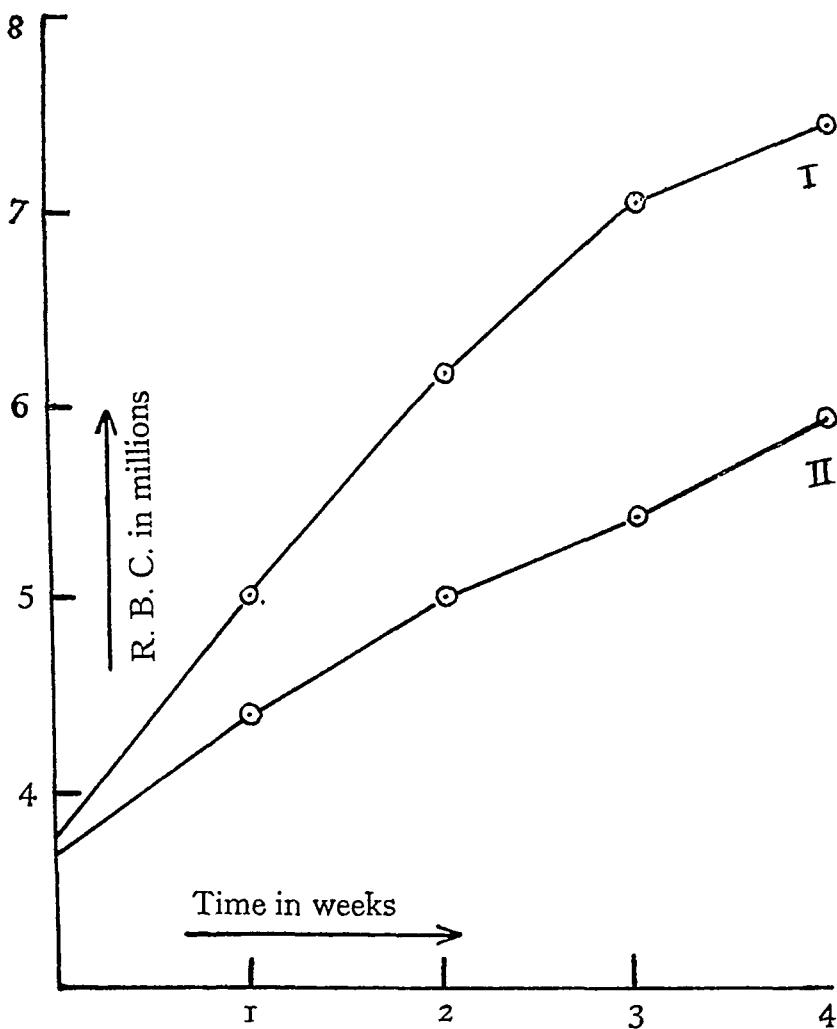
I. Represents the increase in R. B. C. content of the animals receiving 5.0 mg. of nucleic acid *plus* 0.3 mg. or iron.
II. Represents the increase in R. B. C. content of the animals receiving 0.3 mg. of iron only.

FIG. 3
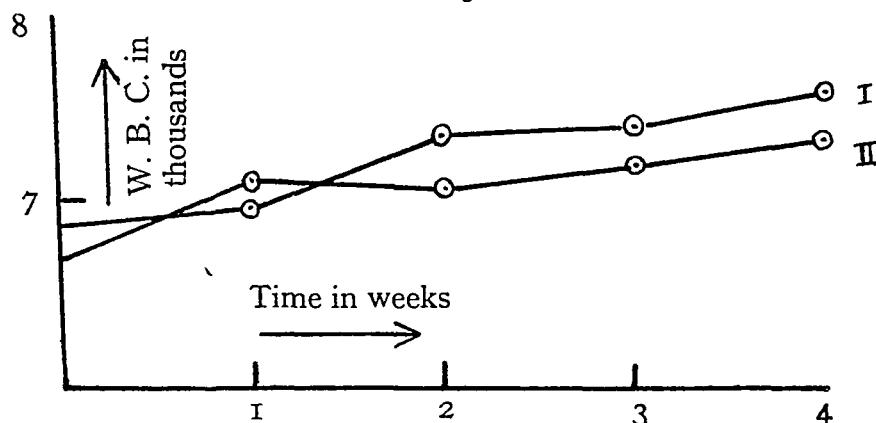
I. Represents the increase in W. B. C. content of the animals receiving 5.0 mg. of nucleic acid *plus* 0.3 mg. of iron.
II. Represents the increase in W. B. C. content of the animals receiving 0.3 mg. of iron only.

SUMMARY

1. A rapid and improved technique for the production of nutritional anæmia in rats has been described which involves the depletion of the animals of their reserve of iron by oral administration of the sodium salt of nucleic acid and subsequent weekly bleeding of the animals by clipping off the tip of the tail.

2. Oral administration of nucleic acid produces no hyperleucocytosis.

3. The blood hæmoglobin and erythrocyte regeneration of the anæmic animals is more rapid if the milk diet is supplemented with nucleic acid and iron than with iron alone.

My best thanks are due to Dr. B. C. Guha for his interest and advise.

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AVAILABLE IRON IN FISH. PART IV. BIOLOGICAL AVAILABILITY
OF IRON IN THE IRON-COPPER-NUCLEOPROTEIN COMPLEX
OBTAINED FROM FISH MUSCLE TISSUE

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In previous publications (1, 2, 3) it has been found that about 30—40
per cent. of the total available iron in fish muscle tissue as estimated by
Hill's method is present as an iron-nucleoprotein complex which resists the
action of pepsin and can be quantitatively estimated by Hill's method as
modified by us (1). This complex iron-bearing substance also contains an
appreciable quantity of copper which can be easily released by 20%
trichloroacetic acid. It was obviously of interest to investigate whether this
iron is biologically active regarding hæmoglobin regeneration in anæmic rats
and mobilization of iron in the body, and whether the copper present plays
any specific rôle in the utilization of iron.

In order to investigate the above question, 24 male adult rats were
made anæmic according to the technique described before (4). When the

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blood hæmoglobin level fell below 45% the animals were divided into six groups having four in each group. The milk diet was used throughout. Prior to the beginning of the actual experiment, the blood hæmoblogin of the individual animal was determined as described previously (5). Each animal of the first group received daily as supplement 0.3 mg. of iron as ferric chloride and two drops of codliver oil. The diet of the second group was supplemented with 0.3 mg. of iron and 0.05 mg. of copper as copper sulphate and that of the third group with 0.05 mg. of copper only per animal per day. The fourth group received a supplement of 0.68 g. of the iron-copper-nucleoprotein complex (corresponding to 0.3 mg. of iron and 0.05 mg. of copper) per animal per day and the fifth group a supplement of 0.689 g. of this complex made free from copper by trichloroacetic acid treatment and washed and dried. The diet of the sixth group was supplemented with 5 mg. of nucleic acid, 0.3 mg. of iron as ferric chloride and 0.05 mg. of copper as copper sulphate. The hæmoglobin determination was made as usual. After the expiry of the fifth week, all the animals were killed and the body iron was determined by the method described previously. The results concerning the hæmoglobin regeneration are shown in Fig. I and Table II. Each curve represents the average results of 4 animals. The total body-iron values are given in Table I.

TABLE I
### *Iron content of the animals as expressed in mg. per 100 g. of body-weight.*

Supplements.	Iron content.	Mean iron content.	Supplements.	Iron content.	Mean iron content.
0.3 mg. of iron as ferric chloride per animal per day.	6.3 6.9 7.3 6.7	6.8	Iron complex corresponding to 0.3 mg. of but free from copper.	4.6 4.9 5.2 4.3	4.8
0.3 mg. of iron as ferric chloride +0.05 mg. of Cu. as copper sulphate.	5.0 5.6 6.1 5.7	5.6	0.3 mg. of iron and 0.4 mg. of copper +5.0 mg. of nucleic acid.	4.2 4.8 3.9 4.6	4.4
Iron-copper-nucleoprotein complex corresponding to 0.3 mg. of iron and 0.05 mg. of copper.	4.2 3.6 4.0 3.9	3.9	0.05 mg. of copper alone.	2.0 1.9 2.6 1.7	2.0

TABLE II

Blood hæmoglobin content of the animals expressed in per cent.

Group No.	Supplements.	Blood hæmoglobin at the end of each week.					
		Initial.	1st week.	2nd week.	3rd week.	4th week.	5th week.
1.	0.3 mg. of Fe per animal per day.	45	58	62	59	66	75
		44	52	53	68	71	73
		49	50	57	60	65	76
		46	52	56	59	66	72
2.	0.3 mg. of Fe +0.05 mg. of Cu per animal per day.	39	44	57	66	74	79
		43	49	62	66	68	72
		47	55	60	76	81	79
		42	48	57	62	69	76
3.	0.05 mg. of Cu per animal per day.	36	44	42	40	38	—
		44	48	45	43	40	—
		50	49	47	46	39	—
		46	47	46	43	42	—
4.	Iron-copper-nucleo-protein complex (0.689 g.) corresponding to 0.3 mg. of Fe+0.05 mg. of Cu per animal per day.	52	68	79	91	96	94
		44	56	70	80	86	97
		43	54	73	86	88	98
		51	62	78	83	90	95
5.	Iron-copper-nucleo-protein complex, corresponding to 0.3 mg. of Fe but free from copper.	50	58	71	78	87	·88
		37	48	62	74	75	85
		51	59	75	81	84	79
		48	55	65	71	79	81
6.	0.3 mg. of Fe+0.05 mg. of Cu+5.0 mg. of nucleic acid.	43	59	74	83	91	97
		45	66	76	82	88	99
		53	64	71	78	83	88
		47	55	68	76	79	86

FIG. 1
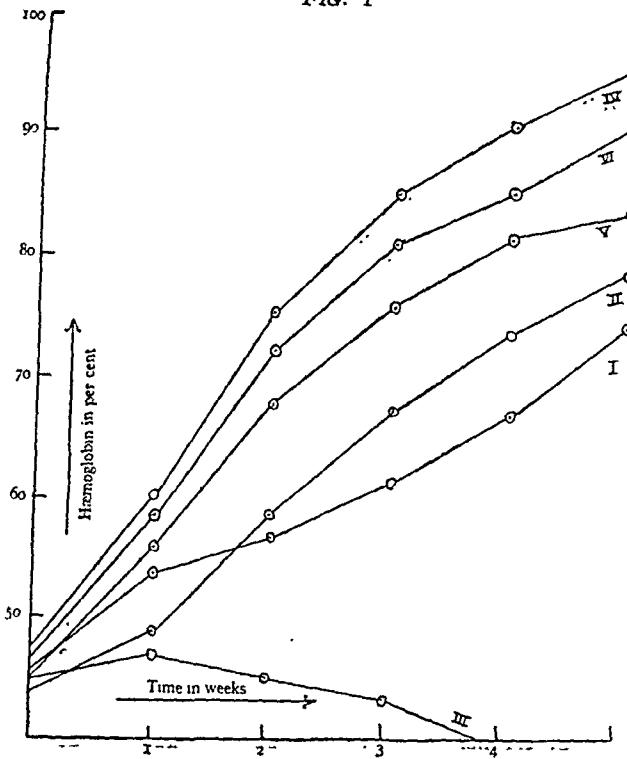
I.   0.3 mg. of iron as FeCl$_3$.
II.   0.3 mg. of iron as FeCl$_3$ + 0.05 mg. of copper as CuSO$_4$.
III.   0.05 mg. of copper alone.
IV.   Iron-copper-nucleoprotein complex corresponding to 0.3 mg. of iron + 0.05 mg. of copper.
V.   Iron-copper-nucleoprotein complex corresponding to 0.3 mg. of iron but free from copper.
VI.   0.3 mg. of iron + 0.05 mg. of copper + 5 mg. of nucleic acid.

DISCUSSION

From the above results it is clear that on administering iron alone to the anæmic animals, kept on whole milk as the basal diet, the rate of hæmoglobin regeneration is slow and incomplete but the absorption of iron in the body is maximum. This seems to indicate that the reduced rate of hæmoglobin formation with iron alone is due to the deficiency of some factor or factors in milk. From this it may be deduced that if this, perhaps, specific factor for hæmoglobin formation is present in the food, the intake of iron or the storage of iron in the body may have effective influence on blood formation.

If, on the other hand, iron is supplemented with copper, more iron is utilized for hæmoglobin building as is indicated by graph II (Fig. 1). The total body-iron in this case is somewhat lower than that obtained with iron

alone. This no doubt exemplifies the partial rôle of copper in the utilization of stored iron for hæmoglobin formation.

When the body-stored iron is reduced to a minimum under anæmic condition, addition of copper alone had no hæmopoietic effect and the animals died. This is, of course, natural, as copper in the absence of iron, can obviously have no hæmopoietic value.

The iron-copper-nucleoprotein complex, as obtained from fish tissue, possesses marked hæmoglobin regenerating capacity. The body-stored iron in this case is below normal, indicating thereby that both the copper and nucleoprotein act as accessary factors for hæmoglobin regeneration thereby making the food-iron more available.

Graphs II and V (Fig. 1) support the findings of Hart and his co-workers (6), Waddel and others (7), Cunningham (8) etc., that copper can be used by rats for hæmoglobin building and that feeding of this element has no effect on the storage of iron in the body. Graph V gives a clearer insight into the specificity of copper regarding hæmoglobin regeneration. Thus by supplementing the iron-copper-nucleoprotein complex to the diet of anæmic rats the average maximum value for hæmoglobin obtained is 94% in the fifth week, whereas if the supplement is made free from copper the average maximum hæmoglobin level rises upto 84% only in the fifth week, with the same iron level. This would further support the view about the specificity of copper in the utilization of iron for hæmoglobin formation.

The marked hæmopoietic effect of the iron-copper-nucleoprotein complex has been further confirmed by graph VI (Fig. 1), which represents the average hæmoglobin value of the animals receiving iron, copper and nucleic acid. Graphs IV and VI (Fig. 1) differ slightly. In graph IV where the natural iron-copper-nucleoprotein complex has been fed, the highest hæmoglobin value is 94%, whereas in graph VI, where pure nucleic acid has been used in addition to equivalent quantities of iron and copper, the maximum value is 88%. This difference, if significant, is probably due to the protein in combination with the nucleic acid which may perhaps play a possitive rôle regarding hæmoglobin synthesis by helping in the formation of pyrrol nuclei necessary for building the prophyrin-moiety of hæmoglobin. The hæmo-poietic stimulation by protein has also been investigated by Benthel (9) who found that a low protein diet followed by excess of iron causes severe anæmia in rats. Abundance of protein and moderate supply of iron produced an actual increase in erythrocyte and hæmoglobin content of the blood. He has further observed that with pregnancy anæmia cases, if a basal whole milk diet was supplemented both with iron and with 65 g. of protein daily, the correction of anæmia and the restoration of the erythrocyte level occurred quite readily, but supplementing with iron alone did not have any appreciate beneficial effect.

The rôle of copper in hæmoglobin synthesis.—One of the chief interests of copper arises from the claim of Hart, Steenbock, Waddel and Elvehjem (6),

that in the presence of this element inorganic iron may be used by rats for hæmoglobin building. Much work has hitherto been done in order to investigate the validity of the above hypothesis. But even at the present day opinion is divided on this point and experimental evidence has been conflicting. But our present investigations appear to support the original conclusion of Hart and his co-workers, regarding the positive effect of copper in hæmoglobin synthesis.

The more interesting problem is about the exact mechanism in which copper stimulates hæmopoiesis. Elvehjem and others (10) have recorded the copper content of different crystalline preparations of hæmoglobin. These analyses were made in an endeavour to answer the question, "Is copper a normal constituent of the hæmoglobin molecule?" Their conclusion, which was in the negative, was not based on their experimental findings but on the fact that a positive result was incompatible with the accepted molecular weight of hæmoglobin. They suggested that the copper is present in loose combination. From our present investigations with the iron-copper-nucleoprotein complex obtained from fish tissue, which has been found to be a normal constituent of all animal tissues so far studied, it seems probable that this substance acts as the precursor for hæmoglobin and that the iron and the associated copper of crystalline hæmoglobin are mainly derived from this substance. It has been shown (graph V) that removal of copper from the iron-copper-nucleoprotein complex diminishes the hæmopoietic power of the latter, which is, however, restored by feeding copper. This would support the view that the iron-copper-nucleoprotein complex is concerned in the formation of hæmoglobin, as we have shown previously that copper is capable of combining with the iron-copper-nucleoprotein at the pH of the stomach (3). The iron and copper of the complex are presumably split off in the alkaline zone of the small intestine, as we have shown before (3) that both iron and copper are released at pH 8.2. The break-down products of the whole complex including those of the nucleoprotein moiety are perhaps used after absorption, in the building of the hæmoglobin molecule. This would seem to lend support to the original idea of Bunge (11) who stated that iron in firm combination with organic compounds, as in hæmatogen present in food was used by animals for hæmoglobin building. The chemical nature of this "hæmatogen", described by Bunge to be an iron-containing organic substance other than hæmatin, was investigated in more detail by Hugouneng and Morel (12). Hæmatogen was regarded by these workers as a prosthetic group in the vitellin molecule and as being a precursor of the hæmoglobin formed during avian embryonic development. This hæmatogen has also been found to contain copper in combination with an iron-phosphoprotein complex. This hæmatogen, which has been found to be present in hen's egg and supposed to be the precursor for hæmoglobin, surely indicates the ready availability of the iron in egg, where no hæmatin compound is present. But Sherman and his co-workers

(13) have found that, although the whole of the iron present in egg can be estimated quantitatively by Hill's method, when fed to anæmic rats it produces only incomplete hæmoglobin regeneration. Hence, it seems likely that with the embryonic development this iron-copper-phosphoprotein is transformed into the iron-copper-nucleoprotein which, as we have shown, leads readily to the formation of hæmoglobin. Thus the more immediate precursor of hæmoglobin seems to be this latter compound, the iron-copper-nucleoprotein complex.

Availability of iron.—We have stated earlier that according to Hill and others the non-hæmin iron, present in foodstuffs, is in the inorganic form and this iron is the only available iron for hæmoglobin synthesis. But in our previous publication (3) we have shown that about 30 to 40% of the tissue iron is present in complex combination with nucleoprotein and copper and this iron can be quantitatively estimated by Hill's dipyridyl method. So Hill's conception regarding the inorganic nature of the non-hæmin iron in foodstuffs does not seem to be justified. Our present investigation indicates further that the hæmoglobin-regenerative capacity of the iron-copper-nucleoprotein complex is much more pronounced than that of the pure iron salt when incorporated in the diet of anæmic animals on the same iron level. This result is contrary to the conclusion of Hill, which seems to be fairly generally accepted, and supports, in a way, as we have mentioned above, the original idea of Bunge who stated that iron in firm combination with organic compounds as in hæmatogen could be used by animals for hæmoglobin building. The iron in the iron-copper-nucleoprotein complex has been found to be present in the ferric state and it has also been shown in our earlier publication that only the ferric iron can combine with nucleoprotein *in vitro*. Now, the further question arises, whether the reduction of iron is necessary before absorption as suggested by Heubner (14), Lintzel (15), Starkenstein and Weden (16), Reimann and Fritsch (17), Moore *et al* (18), and Tompsett (19) or not necessary as postulated by Whipple and Robscheit-Robbins (20), McCance and Widdowson (21), Brock and Hunter (22), etc. This question is under investigation and work concerning the mechanism of absorption of iron is in progress.

SUMMARY

(1) On supplementing iron alone to anæmic rats, kept on whole milk diet, the rate of hæmoglobin regeneration is slow and incomplete but the absorption of iron in the body is considerable.

(2) If iron is supplemented with copper, more iron is utilized for hæmoglobin building.

(3) When the stored iron of the body is decreased to a very low value under anæmic condition, addition of copper alone has no hæmopoietic effect.

(4) The iron-copper-nucleoprotein complex, as obtained from fish tissue, possesses marked hæmoglobin regenerating capacity.

(5) On administering the iron-complex, made free from copper, the rate of hæmoglobin regeneration is slower than when the original complex is fed, which would give further support to the theory regarding the specificity of copper in the utilization of iron in hæmoglobin formation.

(6) The protein content of the iron-copper-nucleoprotein complex may have a positive rôle in hæmoglobin building.

(7) The rôle of copper in the building up of hæmoglobin is discussed.

(8) Hill's conception regarding the inorganic nature of available iron in foodstuffs, which seems to be fairly generally accepted, does not seem to be justified.

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A SENSITIVE CHEMICAL TEST FOR THE DETECTION OF ARGEMONE OIL. PART I. THE TEST AND ITS APPLICATION TO DROPSY-POSITIVE MUSTARD OILS

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Recent publications, specially of R. B. Lal and co-workers on the causation of epidemic dropsy, have aroused considerable interest in this field of investigation. These authors have suggested that argemone oil is the factor responsible for the outbreak of this disease (1). The present author (2), however, pointed out some anomalies of this theory and also reported that a sample of mustard oil prepared in a special way from mustard seeds alone was positive to physical and chemical tests suggested by Lal *et al* (3) for the epidemiologically incriminated mustard oil. The nitric acid test of Lewkowitsch and Warburton (4) for argemone oil has been used extensively for qualitative purposes. It has also been converted into a quantitative one by *Lal et al* (5). On the basis of this test an old sample of proved potent oil (Rangpur oil) appeared to contain about 4% argemone oil (6).

A study of the relevant literature shows that the above *test is not* specific for argemone oil and may be given by a large number of other substances. Hence quantitative figures for argemone oil content on this basis lose much of their significance, unless it is definitely established that no other reacting substance excepting the argemone oil is present.

An attempt has been made by the author to develop a specific and sensitive test for argemone oil, which is described below. The test is at present a qualitative one which enables us to detect the presence of argemone oil even in a concentration of 0.75% in the most convincing manner. In view of its very simple procedure it can conveniently be used in routine work.

Argemone oil when heated with ferric chloride solution in presence of strong hydrochloric acid and ethyl alcohol has been found to give an orange-red precipitate. If the conditions are favourable this precipitate may be obtained as a mass of beautiful orange-red fibrous crystals deposited at the bottom of the test tube or floating in the lower acid layer. They may also be seen collected at the acid-oil interface. Full details for carrying out this test are given below.

EXPERIMENTAL

REAGENTS: 1.   Concentrated hydrochloric acid.

2.   Ethyl alcohol ; Rectified spirit preferable.

3.   Ferric chloride solution prepared by dissolving 10 g. of crystallised ferric chloride ($FeCl_3$, $6H_2O$) in 10 cc. of concentrated hydrochloric acid and 90 cc. of water.

PROCEDURE:
    Two cc. of the oil to be tested is taken in a test-tube, 2 cc. of concentrated hydrochloric acid is then added, the contents mixed thoroughly and then heated in the water-bath kept at 92—95° for 2 minutes. Then 0.8 cc. of ethyl alcohol is added, the mixture shaken thoroughly and kept in the water-bath for 1 minute. Two cc. of ferric chloride solution is then added, the contents mixed thoroughly by shaking and the whole thing afterwards heated in the water-bath for 10 minutes.

    This test was carried out firstly with different dilutions of freshly expressed argemone oil in pure and fresh 'ghani' mustard oil. The observations made are recorded in Table I.

TABLE I

Sample of oil.	Argemone oil content.	Observation.
Pure 'ghani' mustard oil	Nil	No precipitate.
Pure 'ghani' argemone oil	100%	An orange-yellow precipitate just after the addition of ferric chloride solution which becomes deep orange-red and crystalline on heating.
Mixture of argemone oil in mustard oil	10%	No immediate precipitation after the addition of ferric chloride but the orange-red precipitate appears within 2—3 minutes and assumes the characteristic nature later on.
Do	5%	Same as above.
Do	4%	Same as above.
Do	3%	Same as above.
Do	2%	The orange-red precipitate appears in course of heating with ferric chloride solution or a few minutes after the completion of heating. For the characteristic appearance some more time is needed.
Do	1%	Precipitation will be noticed within 2—3 hours or earlier. For the crystalline form keeping overnight is desirable.
Do	0.75%	The orange-red crystalline precipitate is noticed after keeping overnight.
Do	0.5%	No precipitation is noticed.

A slight variation in conditions will not materially affect the performance of the test. It may be noted here that in order to make the test a general one, amounts of reagents necessary to produce good results in case of lower dilutions have provisionally been fixed.

Effects of various factors like storage, light, air and heat have been studied and are recorded below. This has been done in order to meet any objections regarding the validity of the findings with the dropsy-positive mustard oils to be reported later on.

Effect of Storage.—Experiments were carried out by diluting fresh argemone oil with a sample of more than three-year old mustard oil. There was no discrepancy in the result. A sample of argemone oil kept in the

laboratory for more than three years was next tried in different dilutions in fresh and old mustard oil. No important change in the performance of the test could be noticed. It appears, therefore, that storage has little effect, if any, on this test.

Effect of light and air.—The effect of light and air was next studied. Mixtures of argemone and mustard oils were prepared and kept in contact with air in diffused light. A positive test was obtained after one month even when the concentration of argemone oil was 0.75%.

Effect of heat.—Samples of argemone oil heated at 100° to 250° for half-an-hour were tested and nothing untoward could be noticed.

From the above findings it is clear that the various factors mentioned have no marked effects on this test.

Specificity of the test and the nature of the crystalline substance.—A positive test is not given by pure 'ghani' mustard oil—fresh or old. A few market samples, presumably mill oils, were examined and they too were negative to this test. It can therefore be said at present that mustard oil in absence of argemone oil is not likely to give a positive test. However, if there is a precipitate in any case due to some factor or factors other than argemone oil there should be a difference in the appearance of the precipitate and finally in its composition. The crystalline substance formed can easily be separated and recrystallised. It is a define chemical compound and it is very unlikely that compounds from different substances will be the same. So here is a clue for the solution of the difficulty if there arises any in future. Some indications regarding the nature of the crystalline substance have been obtained and a detailed communication on this aspect will be made later on.

Application of the test to dropsy-positive samples of mustard oil. According to the supporters of the argemone theory a sample of epidemiologically incriminated oil should contain about 5-10% argemone oil (7) in order to be able to produce epidemic dropsy like symptoms. It is quite natural, therefore, to expect that the potent samples of mustard oil should be distinctly positive to this test. With this end in view the following reputed dropsy-positive samples of mustard oil were examined very carefully.

1. Proved potent mustard oil—Jamshedpur oil of Dr. Lal obtained through Dr. J. C. Ghosh.
2. Proved potent mustard oil—Sample 'A' from Dr. Lal obtained through Dr. J. C. Ghosh.
3. Proved less potent mustard oil—Sample 'B' from Dr. Lal obtained through Dr. J. C. Ghosh.

Samples A and B were very kindly sent by Dr. R. B. Lal to Dr. J. C. Ghosh on 14.9.38 for some spectrographic work. It may be stated here that Dr. Lal's published data on absorption spectra carried out at that time are on Rangpur and Alamdanga oils—proved potent and proved less potent oil respectively (8). It is quite likely that when sent for similar work at that time they (A and B) may be samples of these two oils.

The above three samples were positive to the nitric acid test but all of them were absolutely negative to the present test. It appears, therefore, that these reputed dropsy-positive samples of mustard oil *do not contain argemone oil even in the concentration of 0.75%*. Under these circumstances we shall have to assume that these samples may contain argemone oil in a concentration less than 0.75% and such oils can produce dropsy-like symptoms or some as yet unknown factor or factors are present in the epidemiologically incriminated mustard oils responsible for the outbreak of epidemic dropsy. The former hypothesis is unlikely in view of the findings of Lal *et al* (9) that 'oil containing less than 1% argemone oil taken daily for 20 days is not likely to produce clinical symptoms'. The latter suggestion will, therefore, hold good even if argemone oil is detected in future in some other samples since the three proved potent samples have all failed to give this test.

Another important observations.—While carrying out the above tests it was noticed that the oily layer in case of dropsy-positive oils was deep black in colour (in some case reddish black) while it was faintly tinted, if at all, in case of 'ghani' mustard oil. Even a three-year old sample of mustard oil properly stored shows very little blackening *under the conditions of the test*. An interesting fact is that the author's sample of mustard oil (2) is also distinctly positive to this blackening test. At present nothing definite can be said excepting only that there are indications of its being useful in future in detecting abnormal samples of mustard oil. Further work is in progress in this direction.

SUMMARY

1. A sensitive chemical test for the detection of argemone oil alone or in mixture with mustard oil has been developed. The test is at present a qualitative one.

2. It has been possible to detect argemone oil in mixture even in the concentration of 0.75%.

3. Factors like storage, light, air and heat appear to have very little effect on the performance of this test.

4. Three reputed dropsy-positive samples of mustard oil of Dr. R. B. Lal failed to give this test showing that *argemone oil is not present in these samples even in the concentration of 0.75%*.

5. The oily layer of dropsy-positive samples of mustard oil under conditions of this test becomes deep black in colour. This observation may be of some use in future.

6. In view of the very simple procedure and the characteristic nature of the test it is recommended for routine work.

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A NOTE ON 'SPAN' IN ANÆMIAS

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Hæmoglobin, the essential constituent of the blood is a pleomorphic compound, as is known by the fact that no two hæmoglobins are quite the same. It is also known that even in the same animal there is more than one component in the hæmoglobin. This is evidenced by the fact that during crystallisation of hæmoglobin different crops of crystals are found at various stages of crystallisation, but 'crystals of the species of any genus belong to the same crystallographic system and generally to the same crystallographic group and they have approximately the same axial ratios, or their ratios are in simple relation to each other, *i.e.*, the hæmoglobin crystals of any genus are isomorphous' and thus, the α, β and γ hæmoglobins may be found in various permutations and combinations in amount in the various species of a genus. And the fundamental difference between these various hæmoglobins can be revealed only by their characteristics absorption spectra.

Going into details of the spectrographic analysis of the hæmoglobins the various absorption bands of hæmoglobin of a particular species at definite gaseous concentrations are found to be occuring at definite wavelengths in the spectrum. Thus for example, the maximum of the α-band of oxyhæmoglobin in man occupies a definite wave-length, namely 5769 A.U., and that of pigeon, 5767 A.U. But if the oxygen of the oxyhæmoglobin is replaced by carbon monoxide the α-band of carboxy-hæmoglobin shifts to the violet end of the spectrum to 5709 A.U. in man and this shift from 5769 to 5709 or 60 A.U. is termed 'Span' by Barcroft (1). This shift or

span is in no way related to the initial position of the α-band of oxyhæmo-globin. Thus though the position of the α-band in both the horse and man is at 5769 A.U., the span in man is only 60, whereas in horse it is 62 A.U.

Though the span is the same in the same animal, it is not so in all the animals of the same species. Even in the same animal, the 'span' is probably constant, only during reasonable variations of the state of health.

That span has a direct relation to the gaseous concentrations of the hæmoglobin has been shown by Barcroft. Hæmoglobin of various animals has different capacities of interaction with gases and the capacity alters and is inversely proportional to the rise of temperature and also to changes in hydrogen ion concentration. The relation to gaseous concentrations is represented graphically thus:

$$Hb + O_2 = O_2Hb + Co$$
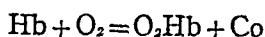
$$\frac{[Co\ Hb]}{[O_2\ Hb]} = K\ \frac{[Co]}{[O_2]}$$

and span is proved to be inversely proportional to K, i.e., span has a definite relation to the gaseous exchanges of hæmoglobin. And gaseous exchanges in health and disease are known to vary. Thus Likarezek et al (2), in the case of man, and in experimental rabbits, have shown that anæmia is associated with a shift of the dissociation curve to the right.

One cannot adequately explain this phenomenon. Probably it may be due to one of the two following causes:

1. Geiger (3), by a process of electrodyalysis, separated from blood two hæmoglobins which had dissociation curves of different degrees of inflection and this has been confirmed by Brinkman and Jonxis (4). Probably in anæmia the relative combination of these two hæmoglobins varies.

2. The absorption spectra of hæmochromogens built on a basis of denatured globin are all the same. It would appear therefore that the denaturisation of the globin reduced all hæmoglobin spectra to a common level. This would go to show that the specific differences in the spectra of different vertebrate bloods found an explanation in the properties of the undenatured globin present and probably there is an alteration in the globin of the hæmoglobin, to account for the shift of the dissociation curve, because it is known that the globins of the adults have no constant composition: Schenck (5) and Lang (6).

Though it is unknown how this phenomenon occurs, one feels justified in assuming that because gaseous exchanges of hæmoglobin are altered in cases of anæmias, the span may show a corresponding change in the various anæmias. If a correlated change is found there is a possibility of classifying anæmias by the measurement of span. The object of the present investigation is to study whether there is any such possibility.
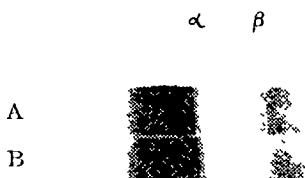
ɔpectra of Oxyhæmoglobin (A) and Carboxyhæmoglobin
(B) to show the shift in α band to the right.

EXPERIMENTAL

Procedure.—Altogether 108 cases of anæmia have been examined. Each oxalated blood specimen is examined under the Reversion spectroscope fitted to a microscope and the reading of the band noted (an average of 10 consecutive readings is taken). Then carbon monoxide (coal gas) is bubbled vigorously for about 5 minutes through the blood sample and immediately the specimen re-examined and the average of 10 readings taken gives the α-band of the carboxyhæmoglobin. The difference between the two readings gives the span in each case.

The results are outlined in the following table. The classification of the various anæmias into hyperchromic, normochromic and hypochromic has been done by taking into consideration the mean corpuscular hæmoglobin. The classification of macrocytic, normocytic and microcytic has been done considering the mean corpuscular volume calculated by the centrifugalisation method. The normals considered are given in the following table.

NAPIER	73 to 105	21.6 to 32.6
WINTROBE	80 to 90	27 to 32
	Mean corpuscular volume in cubic μ.	Mean corpuscular hæmoglobin in αα (one million-millionth of a gramme).

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RESULTS AS ANALYSED BY NAPIER'S CLASSIFICATION.

Hyperchromic					Normochromic						Hypochromic				
Normocytic		Macrocytic		Microcytic	Normocytic		Macrocytic		Microcytic		Normocytic		Macrocytic	Microcytic	
Case No.	Value	Case No.	Value	Case No.	Case No.	Value	Case No.	Value	Case No.	Value	Case No.	Value	Case No.	Case No.	Value
20	0.0515	15	0.063	Nil	1	0.048	9	0.0725	6	0.0535	5	0.0165	Nil	3	0.043
27	0.0535	19	0.0155		2	0.023	10	0.169	41	0.055	16	0.0495		8	0.06
68	0.0625	29	0.028		7	0.034	34	0.021	60	0.0675	25	0.0735		30	0.0345
82	0.058	57	0.0755		11	0.0315	110	0.06	104	0.047	26	0.07		31	0.071
89	0.019	95	0.066		12	0.025					59	0.0625		37	0.06
					13	0.0085					63	0.075		38	0.0875
					14	0.063					78	0.077		39	0.059
					17	0.048					80	0.067		40	0.0125
					18	0.015								47	0.0715
					21	0.062								61	0.0775
					22	0.0255								94	0.055
					23	0.0145									
					24	0.0095									
					28	0.0665									
58	0.0425	86	0.063		32	0.066									
62	0.068	88	0.069		33	0.0145									
64	0.027	90	0.019		35	0.0355									
65	0.063	91	0.072		36	0.033									
66	0.0605	93	0.052		42	0.031									
67	0.0482	96	0.051		43	0.053									
69	0.062	97	0.06		44	0.0755									
70	0.0585	99	0.061		45	0.072									
71	0.0655	100	0.06		46	0.0435									
72	0.051	101	0.056		48	0.066									
76	0.056	102	0.06		49	0.0665									
77	0.068	103	0.066		50	0.0665									
79	0.06	105	0.051		51	0.045									
81	0.053	106	0.05		52	0.067									
83	0.051	107	0.066		53	0.0515									
84	0.068	108	0.047		54	0.052									
85	0.042	109	0.053		55	0.047									
					56	0.0725									

RESULTS AS ANALYSED BY WINTROBE'S CLASSIFICATION.

Hyperchromic						Normochromic						Hypochromic					
Normocytic		Macrocytic		Microcytic		Normocytic		Macrocytic		Microcytic		Normocytic		Macrocytic		Microcytic	
Case No.	Value	Case No.	Value	Case No.	Value	Case No.	Value	Case No.	Value	Case No.	Value	Case No.	Value	Case No.	Value	Case No.	Value
Nil		15	0.063	Nil		7	0.034	1	0.048	35	0.0355	2	0.023	Nil		3	0.043
		19	0.0155			12	0.025	9	0.0725			14	0.063			5	0.0165
		20	0.0515			22	0.0255	10	0.169			17	0.048			6	0.0535
		27	0.0535			36	0.033	21	0.062			18	0.015			8	0.06
		29	0.028			43	0.053	34	0.021			23	0.0145			11	0.0315
		57	0.0755			44	0.0755	50	0.0665			24	0.0095			13	0.0085
		68	0.0625			48	0.066	55	0.047			26	0.07			16	0.0495
		82	0.058			58	0.0425	64	0.027			45	0.072			25	0.0735
		89	0.019			66	0.0605	83	0.051			51	0.045			28	0.0665
		95	0.066			72	0.051	85	0.042			54	0.052			30	0.0345
						88	0.069	90	0.019			62	0.068			31	0.071
						93	0.052	96	0.056			65	0.063			32	0.066
						97	0.06	101	0.051			69	0.062			33	0.0145
						99	0.061	105	0.051			70	0.0585			37	0.06
						102	0.06	108	0.047			76	0.056			38	0.0875
						103	0.066	109	0.053			77	0.068			39	0.059
								110	0.064			81	0.053			40	0.0125
												84	0.068			41	0.055
												86	0.063			42	0.031
												100	0.06			46	0.0435
												106	0.05			47	0.0715
												107	0.066			49	0.0665
																52	0.067
																53	0.0515
																56	0.0725
																59	0.0625
																60	0.0675
																61	0.0775
																63	0.075
																67	0.0482
																71	0.0655
																78	0.077
																79	0.06
																80	0.067
																91	0.072
																94	0.055
																104	0.047

The values have been analysed statistically and correlating the values, no significant difference was found.

Hæmoglobin and Span.—Attempts were also made to find out whether there is any relation between the concentration of the hæmoglobin in the blood and the span. The hæmoglobin in the blood was estimated by means of the Pulfrich photometer in the following way.

0.2 cc. of blood was drawn by means of a pipette. The pipette was then emptied into a measuring flask containing about 15 cc. of distilled water. The pipette was washed out several times with the contents of the flask and finally blown clean. The solution was then shaken with a few drops of ammonia (0.4%) to make the solution clear and transparent. Then sufficient water was added to make the final volume 20 cc. To this solution, sodium hyposulphite sufficient to cover a small knife point was added and shaken well. Then the colour change from bright red to bluish red was complete, a suitable cell (usually 5 mm.) was filled up with the solution and the extinction coefficient determined by means of the Pulfrich photometer using filter S.57 (effective gravity at 5720). The compensating cell was filled up with distilled water. The concentration of hæmoglobin (mg. per 100 cc. of blood) was then obtained by multiplying the observed extinction coefficient by the factor, 16.5 (Zeiss, 7).

To check up the results obtained in the above method, the hæmoglobin concentrations in each case was also determined by Hellige hæmometer and from the tables following, one can see that both the results tally within experimental errors.

The results, as evident from the table below, do not show any relationship between the span and the hæmoglobin concentration out of 44 cases both normal and anæmic.

TABLE III

No.	No. of blood.	Hb by Pulfrich.		Hb by Hellige.	"Span"
1	125	11.0	g.	11.6875	0.063
2	162	8.1	,,	9.075	0.0605
3	68	11.6	,,	12.65	0.0482
4	70	11.98	,,	12.925	0.0625
5	72	11.7	,,	12.375	0.0620
6	73	12.21	,,	12.100	0.0585
7	74	11.2	,,	11.8500	0.0655
8	75	11.97	,,	12.6500	0.051

No.	No. of blood.	Hb by Pulfrich.		Hb by Hellige.	"Span"
9	S.T.M.	14.72	g.		0.066
10	1	13.56	,,		0.055
11	"S"	11.649	,,		0.059
12	218	12.70	,,	12.9250	0.056
13	190	9.57	,,	9.4875	0.068
14	226	9.43	,,	9.9000	0.077
15	227	10.098	,,	10.3125	0.06
16	205	11.10	,,	11.2750	0.067
17	126	12.0	,,	11.5500	0.053
18	139	9.0	,,	9.7625	0.058
19	N/81	12.87	,,	12.375	0.051
20	N/82	11.639	,,	13.3375	0.068
21	N/77	10.23	,,	11,8300	0.042
22	G. K. Roy	13.6	,,		0.061
23	N/83	13.2	,,	13.4700	0.063
24	107	10.98	,,	11.5500	0.069
25	168	10.36	,,	10.7250	0.019
26	170	11.43	,,	13.0625	0.019
27	215	9.159	,,	10.0375	0.072
28	D. N. Mullik	13.4	,,		0.063
29	197	10.068	,,	11.0000	0.052
30	198	10.4	,,	11.2750	0.055
31	159	14.19	,,	14.0250	0.066
32	220	11.5	,,	11.5500	0.051
33	281	12.3	,,		0.06
34	S.T.M.	6.55	,,		0.068
35	235	11.2695	,,	12.2375	0.061
36	H4K	8.547	,,	8.3875	0.06
37	234	10.6755	,,	10.1750	0.056
38	176	8.88	,,	8.8	0.06
39	M	9.83	,,		0.066
40	151	11.286	,,	11.5500	0.047
41	102	10.659	,,	10.1750	0.051
42	162	9.735	,.	9.0750	0.050
43	192	9.60	,,	10.725	0.066
44	237	8.86	,,	9.2125	0.047
45	224	7.67	,,	7.7	0.053
46	179	7.66	,,	6.8750	0.064

SUMMARY

Span is not in any way related to the type of anæmia as evidenced from 110 cases. Amount in hæmoglobin, whether in normal or anæmic blood also bears no relation to the span as is evidenced from an examination of 44 cases.

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NICOTINIC ACID IN BLOOD CELLS

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In a previous communication (Kochhar 1) the author reported that most of the nicotinic acid in blood was present in the red cell and low values for the nicotinic acid content of blood were recorded in diseases associated with changes in erythrocyte such as anæmia. This finding concorded with the conclusion arrived at by Dorfman, Horwit, Koser and Saunders (2) that the growth-promoting activity of nicotinic acid in blood on the dysentery organism was confined to the erythrocyte alone. Data on the distribution of nicotinic acid in blood are of considerable interest in relation to the rôle played by it in the blood, particularly its well-defined functions in metabolism and cell respiration. The present investigation was undertaken to discover quantitatively the comparative distribution of nicotinic acid in various constituents of blood and its variations with continuous intake of 'test doses'.

EXPERIMENTAL

Dogs were chosen for the experiments. Blood samples were obtained from veins, and plasma was separated from the oxalated blood by the usual technique. The cells after removal of plasma were fractionated into two layers, one rich in erythrocytes and the other in leucocytes, by contrifuging at 3500 r.p.m. for about half an hour. The separation of cells in these two layers was, however, not complete, as shown in Table I. The nicotinic acid contents of these layers were determined with the cyanogen bromide-aniline colour reaction of Swaminathan (3,4). Erythrocytes and leucocytes were enumerated in each layer and the nicotinic acid content per erythrocyte 'x' and per leucocyte 'y' was calculated arithmatically. The nicotinic acid

value of samples of blood, calculated from the values given by $(ax+by)$ and plasma, were compared with those obtained by direct tests on whole blood and plasma (Table III).   Blood was taken from the dogs at intervals of 1 hour, 24 hours, 48 hours etc., after giving test doses of 50 mg. of nicotinic acid per kilo body weight.   The results are presented in Tables I, II and III.

TABLE I

Nicotinic acid contents of various fractions of dogs' blood.

Blood Samples	Dogs	Weight in Kg.	Erythrocytes in millions per c.mm.	Leucocytes in thousands per c.mm.	Nicotinic acid μg. per c.mm. $\times 10^{-5}$
(a) Before intake of 'test dose'.					
Erythrocytes layers					
			a x	*b y*	*n*
2	2	11	18.64	4. 7	1629
3	3	12.6	27.66	7. 8	2187
4	4	14	23.98	11. 6	1700
5	5	15	40.04	2. 2	3285
6	6	17	19.71	11. 8	2486
			130.03	38. 1	11287
Leucocyte layers					
2	2	11	15.98	565. 6	2920
3	3	12.6	11.53	199. 8	2480
4	4	14	17.64	139. 4	2300
5	5	15	9.31	608. 4	2820
6	6	17	9.32	119. 5	1444
			63.78	1632. 7	12164
(b) One hour after intake of 'test dose'.					
Erythrocyte layers					
9	2	11	16.70	3.75	1663
10.	4	14	20.12	3.71	1630
11	6	17	22.07	7.43	3130
			58.98	14.89	6423
Leucocyte layers					
9	2	11	12.92	421.87	2350
10.	4	14	12.39	239.93	2800
11	6	17	15.64	138.80	3033
			40.95	800.60	8183

TABLE I (*Contd.*)
Nicotinic acid contents of various fractions of dogs' blood.

Blood Samples	Dogs	Weight in Kg.	Erythrocytes in millions per c.mm.	Leucocytes in thousands per c.mm.	Nicotinic acid μg. per c.mm. $\times 10^{-5}$
(c)	Twenty-four hours after each continuous 'test dose'.				
	Erythrocyte layer				
13	2 2nd day	11	18.18	14.18	2000
14	2 3rd day	11	28.44	12.17	3151
15	2 5th day	11	20.85	8.26	3425
16	4 2nd day	14	29.23	19.14	2780
17	4 3rd day	14	21.08	3.42	2000
18	6 2nd day	17	18.97	5.29	2780
19	6 3rd day	17	18.83	4.79	3200
21	7 2nd day	13	15,20	6.12	1580
22	7 3rd day	13	20.60	3.30	2190
			191.38	76.67	23112
	Leucocyte layers				
12	1	17	15.90	129.86	1422
13	2 2nd day	11	13.90	360.54	2350
14	2 3rd day	11	15.00	295.43	2274
15	2 5th day	11	14.78	681.25	4480
16	4 2nd day	14	6.15	441.48	4509
18	6 2nd day	17	14.09	179.69	2514
19	6 3rd day	17	14.49	170.70	2850
21	7 2nd day	13	10.92	142.70	1500
22	7 3rd day	13	7.91	335.94	2100
			113.14	2737.30	23999

Note:—'Test dose' is 50 mg. per kilo body weight.

TABLE II

Calculation of 'x' and 'y' from Table I.

$x = \mu$g. nicotinic acid per erythrocyte.

$y = \mu$g. nicotinic acid per leucocyte.

Groups of Table I	Erythrocyte in millions per c.mm.	Lecocytes in thousands per c.mm.	Nicotinic Acid -5 $\times 10$	x -11 $\times 10$	y -8 $\times 10$	Range x -11 $\times 10$	Range y -8 $\times 10$
a	130.03	38.1	11287	85.4	39.8	67 — 124	22 — 80
	63.78	1632.7	11964				
b	58.98	14.89	6423	107.7	47.1	79 — 140	21 — 75
	40.95	800.60	8183				
c	191.38	76.67	23112	119.0	37.1	89 — 170	20 — 89
	113.14	2737.30	23999				

TABLE III

DISCUSSION

The results given in Table I show that the nicotinic acid content of blood varies directly with the changes of blood cells of either kind. Erythrocytes and leucocytes are both rich in nicotinic acid but the numerical excess of the former makes them more important in determining the nicotinic acid content of blood. This explains why a low nicotinic acid content of blood in anæmia cases was observed previously. (Kochhar 1).

Nicotinic acid contents per erythrocyte and per leucocyte were calculated from the data in Table I, and the values 85.4×10^{-11} and 39.9×10^{-9} μg were obtained for the individual erythrocyte and leucocyte respectively in the case of normal dogs (Table II). To establish the validity of these observations whole blood, from which layers were obtained in the first instance, was analysed to discover the nicotinic acid content of its various constituents and counts were made for blood cells. These experimental values compared fairly well with those obtained arithmetically from x and y in Table I, and that given by plasma. The nicotinic acid value calculated from any individual constituent of the blood was much lower than the one found experimentally for the whole blood. In normal dogs (group 1, Table III) the erythrocytes, the leucocytes and the plasma contained about 77.2 and 11.9 and 10.9 per cent. respectively of the total nicotinic acid content of blood.

TABLE III

Experimental and calculated values for the nicotinic acid content of blood from which the various fractions described in Table I, were separated.

Blood Samples	Dogs	Erythrocytes in millions per c.mm.	Leucocytes in thousands per c.mm.	Volume of packed cells %	Experimental Plasma μg.%	Experimental Whole Blood μg.%	Calculated Erythrocytes μg.%	Calculated Leucocytes μg.%	Calculated $\frac{100 \times \text{plasma}}{\text{cell vol.}}$ μg.%	Total μg.%	Deviation
(i) Before intake of 'test dose'. Values of x and y are calculated group a, Table I.											
1	1*	6.03	8.43	38	20	414	510	34	13	557	+143
2	2	6.50	32.18	35	150	447	563	87	97	747	+300
3	3	6.74	21.70	39	100	680	539	97	61	697	+17
4	4	6.95	14.21	32	225	844	466	114	145	725	−119
5	5	7.50	45.94	39	131	853	616	150	80	846	−7
6	6	4.20	20.19	33	80	622	522	46	53	619	−3
7	7*	7.64	16.88	37	160	821	650	69	100	819	−2
Total		45.56	159.53	253	1001	4681	3864	597	549	5010	
Mean		6.51	22.79	36	143	669	552	85	78	715	
Percentage							77.2	11.9	10.9		
(ii) One hour after intake of 'test dose Values of x and y are calculated from group b, Table I.											
8	1*	6.03	12.43	32	3710	3182	606	63	2522	3191	+9
9	2†	5.83	34.69	34	281	942	574	96	185	855	−87
10	4	6.33	23.88	33	450	916	503	180	301	984	+68
11	6	4.53	18.89	32	455	978	634	40	309	983	+5
Total		22.78	89.89	131	4896	6018	2317	379	3317	6023	
Mean		5.68	22.47	33	1234	1504	579	95	829	1506	
Percentage							38.4	6.3	55.0		

Notes:—1* Values of x and y are those of the average of their respective groups (Table II).
2† Values of x and y are those of the previous day of the same animal.
3. Numbers of blood samples correspond to those of Table I.

TABLE III (Contd.)

Experimental and calculated values for the nicotinic acid content of blood from which the various fractions described in Table I, were separated.

(iii) Twenty-four hours after continuous intake of 'test dose'. Values of x and y are calculated from group c, Table I.

Blood Samples	Dogs	Erythrocytes in millions per c.mm.	Leucocytes in thousands per c.mm.	Volume of packed cells %	Experimental Plasma μg.%	Whole Blood μg.%	Erythrocytes μg.%	Leucocyte μg.%	Calculated from x and y $\frac{100}{\text{cell vol.}} \times$ plasma μg.%	Total μg.%	Deviation
13	2 2nd day	4.82	34.37	33	260	800	521	81	174	776	— 4
14	2 3rd day	5.34	42.97	34	255	889	587	89	168	844	—445
15	2 5th day	4.66	40.31	35	225	932	760	123	146	1009	+ 77
16	4 2nd day	6.56	25.31	32	250	1111	586	227	170	983	—117
17	4† 3rd day	7.00	19.37	32	220	1066	625	174	149	948	—118
18	6 2nd day	3.78	21.87	30	195	490	578	56	136	770	+280
19	6 3rd day	3.51	22.19	30	200	447	596	55	140	791	+334
20	6* 5th day	3.50	26.25	30	180	409	595	65	126	786	+377
21	7 2nd day	5.23	12.20	35	160	584	543	33	114	690	6
22	7 3rd day	5.75	14.75	38	325	800	607	54	201	861	62
Total		50.15	259.59	329	2270	7528	5998	957	1524	8458	
Mean		5.01	25.96	32.9	227	753	599	95	152	845	62
						71.0	71.1	11.1	17.9		

Notes:—
1.* Values of x and y are those of the average of their respective groups (Table II).
2.† Values of x and y are those of the previous day of the same animal.
3. Numbers of blood samples correspond to those of Table I.

In dogs, after the ingestion of the 'test dose' the nicotinic acid content of plasma increased from its normal mean of 82 μg to 829 μg in 100 cc. of blood in one hour, and then came down to 152 μg in 24 hours—a significant change—whereas only slight increase in the x values was noted, there being no appreciable change in the y values.

Oral administration of nicotinic acid was shown (Kochhar, 5) to produce a sudden rise in the nicotinic acid content of blood and the continuous intake of nicotinic acid resulted in a slight increase in blood content. Table III shows that part of the immediate nicotinic acid excess in blood is due to the increased value of x but most of the increase is due to the increase in the nicotinic acid content of plasma. The slight increase resulting from the continuous intake of nicotinic acid is mainly due to the increased value of x.

In dogs not receiving supplementary nicotinic acid, the erythrocyte contains about 77 per cent. of the total nicotinic acid content of blood and we can safely conclude that slight variation in the leucocyte count will not materially affect the blood values, whereas changes in the erythrocytes in diseases like anæmia will have a pronounced effect on them. It is possible that when rapid destruction of erythrocytes is taking place, as in the hæmolytic type of anæmia, the plasma may become rich in nicotinic acid. But this internal change should not affect the values for the whole blood. However, experiments in the present investigation shown that plasma eliminates its nicotinic acid rapidly. Thus a fall in the blood value must take place

SUMMARY

1. Data on the concentration of nicotinic acid in various constituents of 20 samples of dogs blood are reported. The mean concentration of nicotinic acid in erythrocytes, in leucocytes and in plasma in normal dog's blood has been found to be 77.2, 11.9 and 10.9 per cent. respectively.

2. Thirty-four fractions of blood with known numbers of erythrocytes and leucocytes were analysed. The average nicotinic acid content values per erythrocyte and per leucocyte in normal dogs were 85.4×10^{-11} and 39.9×10^{-9} μg. respectively.

3. Oral administration of nicotinic acid resulted in a slight increase of the nicotinic acid content of erythrocytes. There was a marked increase in the nicotinic acid content of plasma. This increase corresponded to the *peak* found previously.

4. Arithmetically calculated values for nicotinic acid in blood compared well with values found experimentally.

ACKNOWLEDGMENTS

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SULPHAMETHYLTHIAZOLE: CHRONIC TOXICITY AND EFFECT ON HÆMOPOIETIC SYSTEM

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In a previous paper (1) it has been shown that sulphamethylthiazole possesses a definite protective action against pneumococcal (Type 1) infections in mice, and that its activity compares favourably with that of sulphapyridine in this respect. In continuation of this work, investigations have now been carried out on rabbits to determine its absorption and excretion in the system, to study its effect on the blood corpuscles and hæmoglobin, and to find out any other toxic manifestations.

EXPERIMENTAL

Six adult male rabbits were kept separately in metabolism cages. They were given uniform diet throughout the whole experiments with ample quantity of water for proper diuresis. A period of seven days was first of all taken to determine the normal value for the total and differential counts, percentage of hæmoglobin, and to detect any abnormality in the constituents of the urine. After this period of normal observation, sulphamethylthiazole in doses of 100 mg., twice daily, was fed to the rabbits except on Sundays

for a period of two weeks. Thus, the total amount fed for fourteen days was 2.4 g. The blood was examined daily three hours after the morning dose. The whole quantity of 24 hours' urine was collected, and the total excretion of the drug determined.

All the estimations were carried out according to the method of Doble and Geiger (2) slightly modified using diphenylamine as the reagent. As too much alkalinity of urine in rabbits interfered with the development of colour of any sharp intensity, the urine was always made acid to litmus before estimation. Total red and white blood corpuscles, hæmoglobin, and the differential counts were determined daily for each animal. The results in Table I are the average of all the data obtained from the six animals, on each day.

TABLE I

No. of rabbits 6 : Average weight 1.5 kg. : Daily oral dose 200 mg. of sulphamethylthiazole.

Days.	Blood concentration of the drug in mg. per 100 c.c.		Urinary excretion of the drug in mg.		R. B. C. per c.mm. in millions.	W. B. C. per c.mm.	Polymorpho-nuclear leucocytes %	Large mono-nuclear leucocytes %	Lymphocytes %	Eosinophiles %	Hæmoglobin %
	Free	Total	Free	Total							
1	Nil	Nil	20.1	22.7	5.25	6,535	65	2	31	2	89
2	Trace	Trace	28.6	32.9	5.13	6,438	60	3	36	1	85
3	0.18	0.20	38.3	65.7	4.30	5,527	62	2	36	0	81
4	0.50	0.62	35.1	79.2	5.13	4,952	56	4	37	3	84
5	0.86	1.4	45.5	90.2	4.75	5,347	66	2	32	0	92
6	0.93	1.54	51.8	93.2	4.89	5,103	59	1	39	1	89
8	1.13	1.78	69.2	125.8	3,580	4,832	52	3	43	2	94
9	1.32	2.23	59.4	102.0	5,080	4,595	59	1	38	2	89
10	1 59	2.87	65.7	117.9	4,850	4,137	53	4	40	3	86
11	1.62	2.95	82.1	145.3	3,580	3,720	48	3	43	6	86
12	2.14	3.59	71.8	143.4	3,870	4,058	46	3	46	5	84
13	2.5	3.86	87.5	153.8	3,130	3,895	40	8	41	11	75
15	0.21	0.54	162.3	252.5	4,910	3,120	32	6	57	5	78
16	Trace	0.35	40.8	93.9	4,370	3,190	24	3	65	8	80
17	Trace	0.15	35.2	80.3	3,930	3,264	27	3	63	7	77
18	Trace	Trace	25.4	89.2	3,190	4,371	34	4	55	7	75
19	Nil	Nil	39.8	102.3	3,590	4,187	40	5	50	5	77
20	Nil	Nil	40.1	104.2	3,980	4,928	45	4	47	4	79

DISCUSSION

From the table, it is apparent that the toxic effects of this compound are not very marked. There was slight diminution in the total R. B. C. counts and the percentage of hæmoglobin. But the most visible change in the blood picture, was the gradual diminution of the granulocytes with a corresponding increase of lymphocytes. Actually the polymorphonuclear leucocytes diminished to such a great extent, that often 3 or 4 slides had to be examined for the whole field to get a suitable polymorph percentage. But this granulocytopænia was not accompanied by any marked anæmia of the animals. The blood and urine concentration shows a poor absorption and a slow excretion of the drug. In none of the animals the blood concentration rose more than 3 mg. of the free drug per 100 cc. The conjugation in both blood and urine was fairly high ; sometimes as much as 60—80 per cent of the drug was excreted in the urine as conjugated. This shows that rabbits rapidly and to a considerable extent, acetylate the drug for excretion (3).

Granulocytopænia is alone a factor, apart from all others, which should be taken into account for the evaluation of the chronic toxicity of any compound. A high degree of acetylation also brings about a diminution of the therapeutic efficiency. Of course, acetylation varies in different animals, and with that the therapeutic efficiency. Considering all these factors, it may be said that the value of sulphamethylthiazole is limited at least in the case of rabbits. Though very effective in mice (1) its tendency towards chronic toxicity in rabbits in the form of agranulocytosis will restrict its prolonged use in acute infection.

SUMMARY

1. Low concentration of the drug in blood and slow excretion in urine were noticed by daily feeding 200 mg. of sulphamethylthiazole to rabbits.

2. The drug underwent conjugation both in blood and urine to a considerable extent.

3. Great diminution of granulocytes with corresponding increase in lymphocytes was observed.

4. No marked anæmia developed.

5. The use of the drug should be restricted for its chronic toxicity.

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ON PLANT PHOSPHATASES. PART III* GLYCERO- AND PYROPHOSPHATASES AND PHYTASE ACTIVITIES OF PLANT TISSUES

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Phosphatases are distributed in various parts of dormant and growing plants as well as in dormant and germinating seeds. The enzyme is found not only in great majority of seeds (Namec, 1) and leaves (Burkard and Neuberg, 2 ; Baba, 3 ; Ignatieff and Wasteneys, 4), but also in roots (Ignatieff and Wasteneys, 4) and tubers like potato and sugar beet (Pfankuch, 5) and in the fruits like banana (Dale, 6).

It is generally known that the phosphatase activity of higher plants varies with the stage of the growth of the plant. Ignatieff and Wasteneys (4) studied the changes in the phosphatase content of bean, potato, radish and wheat plants, and found that the phosphatase activity in the dormant bean seed was low, and that the embryo contained higher concentrations of the enzyme than the cotyledons. In the germinated seed the concentration of the enzyme was found to be greater than in the dormant seed. The hypocotyl and the stem had the greatest concentration of the enzyme, but its concentration was found to diminish after a short period of growth. The concentration of the enzyme in the leaves was found to be higher than that of any other part of the plant throughout its life history. In the leaves, the highest phosphatase activity appeared to be associated with the maximum rate of growth and decreased as the plant reached maturity. Since the highest phosphatase concentration in the plant was found in the leaves, the site of vigorous carbohydrate metabolism, the authors have suggested that phosphatase and phosphates play an important rôle in carbohydrate metabolism of the higher plant. Ignatieff (7) continued his studies on the possible relationship between the phosphatase activity and carbohydrate metabolism in plants. His observations on the distribution of phosphatase activity and

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*Parts I and II of this series appeared in *Z. physiol. Chem.*, 1937, **245**, 185 and 1938, **254**: 126.

analysis of growth in "Canada Wonder" bean have shown that there exists a close correlation between the increase of dry matter in the plant and the phosphatase activity of the leaves. These findings further confirm the view that phosphatase plays a rôle in carbohydrate metabolism in plants. In view of the importance of the above and the need for further knowledge regarding the distribution of phosphatases, which hydrolyse various phosphoric compounds widely distributed in plants, the present study was undertaken with a view to (1) demonstrate the presence of enzymes which hydrolyse glycerophosphate, pyrophosphate and phytase into inorganic phosphorus in several plants, and their distribution in vegetable tissues, and to (2) establish the viewpoint that glycero- and pyrophosphatases are different enzymes.

Glycero- and Pyro-phosphatase Systems of Plant and Animal Tissues.—Ever since the discovery by Lohmann (8) of the presence of pyro-phosphate in plants, yeast and muscle, attention has been devoted to the study of the enzyme pyro-phosphatase which is present in several plant and animal tissues. Neuberg and Wagner (9) were the first to show that diphenyl pyrophosphate in the form of its potassium salt is hydrolysed both by the phosphatase of takadiastase and by that of horse kidney. It was of interest, therefore, to many workers to know whether there is any relation between the enzyme which hydrolyses pyrophosphate and the phosphoric esterases widely distributed in the tissues, which hydrolyse glycerophosphate, hexosediphosphate, nucleotides and many other phosphoric esters.

Kay (10) found that the order of activity displayed by various mammalian tissue extracts was the same for glycerophosphate as for pyrophosphate. Further, he showed that the ratio of the amount of orthophosphate P produced from excess glycerophosphate and pyrophosphate was approximately constant from one tissue to another. He suggested, therefore, that the enzyme responsible for the hydrolysis of glycerophosphate was identical with the enzyme which hydrolyses pyrophosphate to orthophosphate. Jacobson (11) found that glycerophosphatase and pyrophosphatase cannot be separated from one another by inactivation. Roche (12) from his studies on blood phosphatase has shown that different enzyme preparations hydrolyse pyrophosphate at different rates. He, therefore, suggested that a specific pyrophosphatase may be present in blood. In favour of the non-identity of the two phosphatases is the claim of Japanese workers (13, 14) to have isolated a pyrophosphatase free from other phosphoesterases. Takahashi (13) has shown that kidney contains at least two pyrophosphatases which in presence of their activators hydrolyse diphenyl pyrophosphate optimally at pH 4 and pH 9 respectively to orthophosphate. He has suggested the existence of three types of pyrophosphatases with activity optima near pH 4.5 and 9 respectivecly. Munemura (15) has prepared all the three types of pyrophosphatases free from one another. Bauer (16) isolated from yeast

extracts by heat inactivation, enzyme solutions which are active towards pyrophosphate, but inactive on   α-glycerophosphate.   Hence a specific pyrophosphatase which is not identical with glycerophosphatase and adenylpyrophosphatase is shown to exist in yeast extracts.   In a later communication Bauer (17) has reported the preparation of α-glycerophosphatase free from pyrophosphatase by adsorption on Al_2O_3 (C γ).   The individuality of the two enzymes is thus established.

From a study of the relative stability of the two enzymes at different pH, Giri (18) has obtained evidence to show that the pyrophosphatase present in the aqueous extracts of germinated soya bean (*Glycine hispida,* black variety), is quite distinct from the glycero-phosphatase.   Fleury and Courtois (19) have shown that takadiastase is richer in glycerophosphatase than in pyrophosphatase.   An inverse distribution occurs in emulsin. Experiments on heat inactivation of the two enzymes have shown that they differ in their stability, a fact which was established before by Giri (18).

It is quite clear from the foregoing findings of several workers that the two enzymes are not identical.   If, on the other hand, the two enzymes are identical we have an unusual case of enzyme specificity, namely an enzyme which can hydrolyse at once an organic substrate like glycerophosphate and an inorganic substrate pyrophosphate.   From the point of view of our knowledge of the mechanism of enzyme reactions the non-identity of these two enzymes is of great importance.   In the present investigation fresh evidence is presented pointing to the non-identity of the two enzymes.

EXPERIMENTAL

The cereals, tubers, leaves and vegetables listed below were treated as follows for the preparation of active extracts.

Cereals, Oil Seeds and Nuts.—The cereals were powdered well and passed through a 60-mesh sieve.   In some cases the seeds were defatted and powdered.   Defatted castor seed powder was prepared by crushing the seed after removing the shells, and extracting the oil by pressing it at 2000 lbs. pressure in a hydraulic press.   The seed cake was then extracted with low boiling petroleum ether (b.p. 30°-50°) for 30 hours in a Soxhlet extractor. The dry fat-free material thus obtained was powdered well and passed through 60-mesh sieve.   Groundnuts were similarly defatted.   Other nuts were used for extraction without any such treatment.

Tubers and Vegetables.—Tubers were ground fresh in a mincing machine, and the shredded pulp was immediately used for extraction.

Leaves.—Fresh leaves were similarly treated, and the shredded pulp was directly used for the extraction of the enzymes.   In some cases (sandal leaf) the leaves were dried by desiccating over anhydrous calcium chloride and powdered.

PREPARATION OF ACTIVE EXTRACTS

Cereals, Oil Seeds and Nuts.—Ten g. of the powder were extracted with 50 cc. of toluenated water for 24 hours at room temperature (26-28°) and filtered. The filtrate was directly used for the determination of the activity. In the case of seeds (soya bean and castor bean) which contain high concentrations of the enzyme, the volume of the water used for extraction was proportionately increased.

Tubers, Vegetables and Leaves.—The same procedure was adopted for the preparation of active extracts from vegetables, tubers and leaves.

The moisture contents of the materials were determined and from these values the activity of the enzyme contained in the extract, which corresponded to 1 g. of the dry material was calculated. The figures given in Table I represent the activities of the phosphatases contained in the aqueous extracts, which corresponded to 1 g. of the moisture-free material.

Determination of the Activities of the Enzymes.—The activities of glycero-pyrophosphatases and phytase were determined at pH 5.2 and at temperature $35 \pm 0.1°C$. The reaction mixture always contained 10 mg. of total phosphorus irrespective of the substrate used. The reaction mixture contained 10 cc. of acetate buffer pH 5.2 ; substrate containing 10 mg. phosphorus ; enzyme and water to make up the total volume to 25 cc. The reaction was allowed to proceed for one hour in the case of glycero-and pyro-phosphatases and 6 hours in the case of phytase activity determinations. Phosphorus was determined in 10 cc. of the reaction mixture and from the value thus obtained (mg. P) the activity of the phosphatases (expressed in mg. inorganic phosphorus formed in 10 cc. of reaction mixture) contained in the total volume of the extract corresponding to 1 g. of the original dry material, was calculated, and the values thus obtained are presented in Table I.

Substrates.—Merck's crystalline sodium β-glycerophosphate was used for the determination of the activity of glycerophosphatase.

Sodium pyrophosphate was prepared by heating Sorensen's secondary sodium phosphate to a red heat in a platinum dish.

The substrate used for the determination of the activity of phytase, was the sodium salt of inositol hexaphosphoric acid ($C_6H_{12}O_{24}P_6Na_{12} + 44H_2O$). This compound was prepared by the method of Posternak (20) with slight modification.

Preparation of the Sodium Salt of Inositol Hexaphosphoric Acid.—Five g. of phytin (Kahlbaum) were dissolved in 20 cc. of N/2 hydrochloric acid, and ferric chloride solution was added until a persisting yellow colour was formed. A white precipitate of the ferric salt of inositol hexaphosphoric

acid was formed, while the inorganic iron phosphate remained in the acid solution. The ferric salt was removed by filtration, washed repeatedly with water and a homogeneous suspension of it was made. Pure N-sodium hydroxide solution was slowly added to the suspension of the ferric salt under constant stirring, until a drop of the mixture when placed on a filter paper, formed a brown spot with a colourless edge. It was then filtered and washed. To the filtrate was added half its volume of alcohol and kept in an ice chest over night. A syrupy mass was formed at the bottom. The alcohol was decanted and the crystalline viscous mass was again dissolved in water and heated on a water-bath, in order to drive off traces of alcohol, and excess of water. On cooling the sodium salt of inositol hexaphosphoric acid crystallised out. The pure salt was dissolved in water and used as substrate for the phytase activity determination. The total phosphorus content of the solution was determined and diluted to contain 10 mg. phosphorus in 5 cc. of the substrate solution.

TABLE I

Glycero- and Pyro-phosphatases and Phytase Activities of Plant Tissues.

No.	Name of the plant product	Botanical name	Activity			Ratio Pyro-/Glycero phosphatase
			Glycero-phosphatase	Pyro-phosphatase	Phytase	
	Gereals					
1.	Maize	*Zea mays*				
	(a) Milky stage		0.420	1.09	0.167	2.6
	(b) Ripe grain		0.239	0.648	0.064	2.7
2.	Rice	*Oryza sativa*	0.311	0.667	0.059	2.1
3.	Cholam	*Sorghum vulgare*	0.346	1.076	0.073	3.1
4.	Cambu	*Pennisetum typhoideum*	0.247	0.856	0.174	3.4
5.	Ragi	*Eleusine Coracana*	0.042	0.203	0.010	4.8
6.	Barley	*Hordeum vulgare*	0.099	0.350	0.042	3.5
	Pulses					
7.	Bengal gram	*Cicer arientinum*	0.085	0.735	0.043	8.6
8.	Black gram	*Phaseolus mungo*	0.493	1.221	0.043	2.5
9.	Green gram	*Phaseolus radiatus*	0.910	2.580	0.180	2.8
10.	Cow gram	*Vigna catiang*	0.284	1.168	0.128	4.1
11.	Field bean	*Dolichos lablab*	0.351	1.272	0.152	3.6
12.	Horse gram	*Dolichos biflorus*	0.433	2.10	0.097	4.8
13.	Peas	*Pisum sativum*	0.162	0.982	0.132	6.0
14.	Soyabean	*Glycine hispida*	0.600	2.05	0.210	3.4
15.	Red gram	*Cajanus indicus*	0.278	1.064	0.104	3.8

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TABLE I (*continued*)

| No. | Name of the plant product | Botanical name | Activity | | | Pyro-Ratio |
			Glycero-phos-phatase	Pyro-phos-phatase	Phytase	Glycero phos-phatase
	Oil Seeds and Nuts					
16.	Groundnut (defatted)	*Arachis hypogea*	1.41	2.66	—	1.9
17.	Gingelly seed	*Sesamum indicum*	0.185	0.443	0.139	2.4
18.	Almond	*Prunas amygdalis*	0.128	0.398	0.065	3.1
19.	Cashew nut	*Anacardium occidentale*	0.117	0.339	0.075	2.9
20.	Walnut	*Juglans regia*	0.050	0.114	0.070	2.3
21.	Brazil nut	*Bertholletia excelsa*	0.040	0.080	0.076	2.0
22.	Mustard seed	*Brassica juncea*	0.040	0.322	0.124	8.0
23.	Cocoanut	*Cocos nucifera*	0.030	0.120	0.016	4.0
24.	Castor seed (defatted)	*Ricinus communis*	2.34	8.94	—	3.2
25.	Cotton seed	*Gossypium herbeceum*	0.506	1.84	—	3.6
	Roots and Tubers					
26.	Radish (white)	*Raphanus sativus*	1.380	4.51	1.69	3.3
27.	Potato	*Solanum tuberosum*	1.180	2.50	0.125	2.1
28.	Carrot	*Daucus carota*	2.70	5.97	1.16	2.2
29.	Sweet potato	*Ipomoea batatas*	3.03	4.65	0.600	1.5
	Vegetables					
30.	Cluster bean	*Cyamopsis psoralioides*	2.7	7.03	0.703	2.5
31.	French bean	*Phaseolus vulgaris*	0.481	0.830	0.201	1.7
32.	Ridge gourd	*Luffa acutangula*	8.02	23.08	2.7	2.9
33.	Snake gourd	*Trichosanthes anguina*	10.9	30.6	2.0	2.8
	Leaves and Leafy Vegetables					
34.	Amaranth	*Amaranth gangeticus*	1.06	2.7	0.530	2.5
35.	Cabbage	*Brassica oleracea-capitata*	1.87	5.07	0.710	2.7
36.	Fenugreek	*Trigonella foenumgraecum*	3.66	13.15	2.77	3.6
37.	Sandal leaf (desiccated)	*Santalum album*	2.15	7.92	—	3.7

The results show that the enzymes, glycerophosphatase, pyrophosphatase and phytase are widely distributed in plants. Fresh vegetables, tubers and leaves are rich sources of these enzymes. The activities of all phosphatases decrease during ripening of seeds (maize). Further it can be seen from the results that the ratio, (*Activity of pyrophosphatase : Activity of glycerophosphatase*) varies widely from one plant to another. It is, therefore, clear that this wide variation implies that the two enzymes are distributed in plants in various proportions and hence the enzymes are not identical. Further the ratio is always found to be higher than one in all the plant materials studied.

Glycero- and Pyro-phosphatase Activities of Animal Tissues.—Glycerophosphatase is found in a number of animal tissues and is invariably accompanied by the enzyme pyrophosphatase as shown by Kay (10). According to Kay these two enzymes are always present in the same proportions. For purposes of comparison with the glycero-pyrophosphatase systems in plants, the ratios of the activities of the two enzymes from different tissues of animals and body fluids as observed by a number of workers are tabulated below.

TABLE II

Glycero- and Pyro-phosphatases of Animal Tissues.

Tissues	Activity of glycero-phosphatase	Activity of pyro-phosphatase	Ratio: Pyro/glycero-phosphatase	Author
Duodenal mucosa, bone, kidney, lung, liver, muscle extract, blood plasma ...	—	—	0.5	Kay (10)
Prostate extract ...	2.49	0.26	0.105	Kutscher and Wolbergs (21)
Human milk ...	0.540	0.260	0.481	Giri (22)

It can be seen from the above table that the ratio: pyro-/glycero-phosphatases of animal tissues and body fluids is always less than one.

DISCUSSION

The varying extent of the hydrolysis of glycero- and pyro-phosphates by the extracts of various plant tissues support the view that there is a specific enzyme for pyrophosphate hydrolysis, and that the two enzymes,—glycerophosphatase and pyrophosphatase—are different.

A significant point brought out by these experiments is the difference in the ratio of the activities of the two enzymes occurring in plants and animals. The pyro-glycerophosphatase system occurring in plants is characterised by a high ratio of pyro-/glycerophosphatase activities, which is found to be always higher than one, while the ratio of the activities of the system existing in animal tissues is always found to be less than one. This characteristic difference between the two systems occurring in plants and animals may be usefully employed as a means for determining the origin of the phosphatase.

A knowledge of the relation between the activity of phosphatases and other metabolic processes in plants is of great importance. There may be a relationship between phosphatase activity and photosynthesis in plants, as the enzyme is more concentrated in those parts of the plant in which photosynthesis is most active. Ignatieff and Wasteneys (4) observed that the concentration of phosphatase in the leaves is higher than that of any other part of the plant throughout its entire life period. In the present investigation a similar observation is made namely the phosphatase activity of leaves is very high. Another point of interest arising out of the present study is that defatted oil seeds contain high concentrations of phosphatase than other cereals rich in proteins and starch. The activity of the extracts obtained from other oil seeds and nuts, from which the oil has not been removed, was found to be low compared to those obtained from defatted seeds. This is probably due to the incomplete extraction of the enzyme contained in the seeds.

The presence of phosphatase in relatively large concentrations in leaves and tissues of high metabolic activity, invites speculation as to the role which this enzyme may play in the metabolism of plants. The possible relation between alcoholic fermentation of sugar by yeast extracts on the one hand, and the respiratory sugar breakdown in plants on the other, has been the subject of intense study by several workers. It is known that phosphates stimulate the respiratory sugar metabolism in higher plants and it has been suggested that phosphoric esters are formed as intermediate compounds. Bodnar (23,24) demonstrated for the first time that phosphorylation takes place when inorganic phosphate was added to ground peas in presence of toluene. Zalesky and Pissarjewski (25) and Rao (26) confirmed

the findings of Bodnar. These findings indicate the possible parallelism between carbohydrate metabolism in the higher plants and in yeast or muscle. Tanko (27) investigated the optimum conditions of the experiments described by Bodnar (24) and isolated the hexosediphosphate and hexosemonophosphate esters which were formed from added inorganic phosphate by pea preparations in the presence of toluene, without any extra carbohydrate. Dale (6) demonstrated that during the ripening process of banana, both fructose diphosphate and hexosemonophosphate are present. According to Pfankuch (5) the function of potato and beet phosphatases is to control carbohydrate metabolism, in which phosphorylation plays an important part, and he, therefore, suggests that the phosphatase serves as a break on the degradation of carbohydrates. It appears, therefore, that further knowledge of the role of phosphatases in plants in their relation to carbohydrate metabolism, is desirable.

SUMMARY

The activities of glycero- and pyro-phosphatases and phytase of various plant materials have been determined.

In leaves, fresh vegetables and tubers, the concentration of the enzyme is found to be much greater than those in seeds rich in proteins and starch.

The ratio of the activities of pyro-/ glycero-phosphatases observed in a large number of plant materials varies widely from one plant to another. The ratio of the activities of the two phosphatases occurring in plants is always found to be more than one, while that for the system existing in animal tissues and body fluids, it is found to be less than one.

The possible rôle of phosphatases in plant metabolism is discussed

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THE STATE OF VITAMIN C NUTRITION IN PULMONARY TUBERCULOSIS

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Although considerable work has been carried out on this subject it was thought that further work was desirable from the point of view of the very important bearing that it has on the well-being of the community. It was also our object to bear upon the problem an attack from as many different angles as possible. Groups of workers (Harris and Ray, 1 ; Heise and Martin, 2 ; Schroeder, 3 ; Rothstein, Ratish and Harde, 4 ; Abbasy et al, 5 ; Thaddea and Hoffmeister, 6 ; Hurford, 7 ; Bumbalo and Jetter, 8, 9 ; Weber, 10 ; Baksh and Rabbani, 11) have reported a diminished excretion of Vitamin C in pulmonary tuberculosis. We have estimated the total excretion of Vitamin C in urine by Harris and Ray's modified method (12) over a period of 48 hours in 60 pulmonary tuberculosis cases and found that 93.4% of the patients are in a state of vitamin C subnutrition, taking 13 mg. as the physiologically minimum urinary output daily (Table I).

Abbasy et al (12) opine that the 'saturation test', or the response to a 'test dose' of 70 mg. of ascorbic acid per stone of body-weight on one or more consecutive days, constitutes a surer measure of the state of vitamin C nutrition. Göthlin (13) also remarks that "the saturation procedure has the advantage that it re-acts to less pronounced C-deficiency, and that, in the hands of an experienced investigator, it gives more finely graded results." Saturation experiments have been conducted in the Medical College Hospital, Patna on 60 cases of pulmonary tuberculosis in two sets. Seventy mg. of synthetic vitamin C ("Redoxon" of Hoffmann-la-Roche) per stone of body-weight was administered on three consecutive days to 30 cases (7 females and 23 males of adult age). A second group of 30 cases (5 females and 25 males of adult age) were given an equivalent amount of natural vitamin C in the

form of sundried pulp of Indian gooseberry (*Emblica officinalis*). Following upon Giri and Doctors' (14) discovery that dried *Emblica officinalis* pulp is unusually rich in vitamin C we have devised an easy method of its preparation which yields a powder very rich in vitamin C (2.5%). The results are given in Table II.

DISCUSSION

From a careful analysis of the diet of the patients in the Hospital, it transpired that the food consumed was fairly rich in vitamin C, containing as it usually did a sufficiency of fresh fruits, particularly oranges. Thus the need of vitamin C in tuberculosis is greatly increased due to its increased destruction caused probably by pyrexia, toxæmia and hyperidrosis, if any.

Saturation experiments conducted in America show that 1.0 to 1.9 mg. of vitamin C per kg. of body-weight daily is required to maintain the tissues in a state of complete saturation (15, 16). Abbasy *et al* (5) have shown that the response of pulmonary tuberculosis cases to the 'test doses' of vitamin C was negligible in active cases, intermediate in moderate and good in quiescent ones. Bumbalo and Jetter (8) reported that tuberculous children required 1000 to 1200 mg. of ascorbic acid to produce any significant increase in the urinary output of vitamin C. Wernicke (17, 18) has conducted saturation experiments on lupus cases.

An interesting result of our investigation has been that after the administration of natural vitamin C in the form of *Emblica officinalis* powder, a greater number of pulmonary tuberculosis patients showed saturation as well as evidence of minimum physiological intake of vitamin C. This better result may be attributed to a factor or factors present in gooseberry pulp and absent in synthetic vitamin C. We are inclined to think that the additional factor may possibly be vitamin P (Bentsáth *et al*, 19) which according to Bentsáth and Szent Györgyi (20) governs the capillary fragility in association with vitamin C. The exact mechanism of the action of any possible vitamin P present in gooseberry pulp is not at present clearly understood. A tentative explanation is that the patients' capillary resistance is increased which may help the excretion of a greater amount of vitamin C due to a comparatively smaller destruction and conversion in the body. Alternatively it is possible that vitamin P modifies and enhances the action of vitamin C. It may be mentioned here that Adant (21) could not get increased capillary resistance by oral administration of vitamin C alone and others (Jerlid, 22 ; Scarborough, 23, 24 ; Elmby and Warburg, 25) have corroborated it. Further in contradistinction to what Harris and Ray said, no direct relationship of the urinary response after 'test doses' to the 'resting level' of vitamin C in urine could be obtained. Similar results have been reported by Ranganathan and Sankaran (26) and by Chakravorty and Ray (27).

✓ The urinary excretion of vitafin C after 'test doses' also bears no relationship with the clinical condition of the patient or the anatomical extent of the lesions as seen by X-rays.

TABLE I

Total urinary excretion of vitamin C per 24 hours in pulmonary tuberculosis

Group	No of patients.	Total excretion.	Remarks.
I	15	0—5 mg.*	Severe subnutrition
II	41	6—12 mg.	Moderate subnutrition
III	4	13 mg. and above.†	No subnutrition.

†Maximum excretion, 17.20 mg.
*Minimum excretion, 0.95 mg.

TABLE II

Saturation experiment

Expt.	Showing evidence of minimum physiological intake.		Saturation after 'test doses' for					
			1 Day.		2 Days.		3 Days.	
	No. of patients.	Per cent.	No. of patients.	Per cent.	No. of patients.	Per cent.	No. of patients.	Per cent.
Re-doxon.	3	10	0	0	0	0	1	3.3
Indian gooseberrry.	14	46.7	0	0	1	3.3	5	16.6

Approximately 1500 mg. of vitamin C was ingested before any significant rise in the urinary output occurred in 45% of cases (above 20 mg.). In view of the observation of Scarborough and Stewart (28) and of Ghua and Sen Gupta (29) that part of the ascorbic acid in urine is excreted in the combined form, the vitamin C nutrition in pulmonary tuberculosis would merit re-investigation.*

We wish to thank Messrs. Hoffman La Roche and Co. for a generous gift of 'Redoxon' tablets.

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*After the data were completed and the manuscript was ready for the press, two papers of Banerjee *et al* (30-31) appeared in which the authors show that in pulmonary tuberculosis the amount of combined ascorbic acid excreted in urine is proportionately much higher than in normal persons.

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NEW HÆMOSTATIC AGENTS. PART I. EXPERIMENTS WITH AYAPANIN AND AYAPIN

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Eupatorium Ayapana, Vent. (=*E. triplinerve,* Wahl.), a native of Brazil, has long been naturalized in India. The leaves and twigs are extensively used as an indigenous remedy in many parts of the world. The aromatic herb is supposed to be toxic, expectorant, diaphoretic, antiperiodic and aperient. It is said to be a valuable remedy against ague. Internally it is also given as an antidote to snake bites. In Europe the dried leaves of the plant had once been largely used as a tonic under the name of 'Ayapana tea'. In Bengal the pressed juice of the fresh leaves is used as a hæmostatic agent, particularly for checking internal hæmorrhage.

A number of investigations has been carried out from time to time with a view to isolate the various chemical constituents of the plant. Dymock, Warden and Hooper (1) reported the presence of an essential oil, and a neutral crystalline substance to which the name 'ayapanin' was given.

Recently Nag and Bose (2) isolated from the dried leaves of the plant, a crystalline constituent, $C_{12}H_{10}O_4$, m.p. 110—112°, which was also called ayapanin, although it gave a characteristic colour reaction different from that reported by Dymock· *et al.* Bose and Roy (3) isolated two different neutral crystalline constituents from the same source. One of these, for which the name ayapanin was retained, was found to be identical with herniarin or 7-methoxycoumarin (I) isolated from *Herniaria hirsuta* (Barth and Herzig,

4). The second compound, named ayapin has been shown to be 6:7-methylenedioxycoumarin (II) (Bose and Ghosh, 5: *cf.* also Spath, Bose and Schläger, (6).
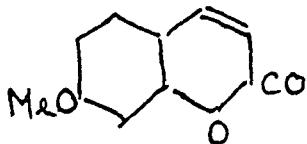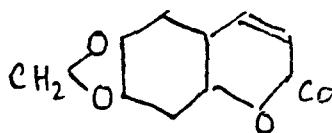
$$\textbf{(I)} \qquad\qquad\qquad\qquad \textbf{(II)}$$

Having ascertained the constitution of these substances we thought it desirable to investigate whether the well-known hæmostatic properties of *E. Ayapana* are associated with these two natural coumarins. A number of experiments carried out with them, both *in vitro* and *in vivo*, definitely proved that they (I and II) possess remarkable blood-coagulating properties (*cf.* Bose and Sarkar, 7). The maximum lowering in the coagulation time of rabbit's blood has been found to be of the order of 50, when calculated from the formula,

$$\text{H.A.} = \frac{(n-o)}{n} \times 100$$

Where H.A. = Hæmostatic activity or extent of lowering in clotting time.
　　　　n = Normal clotting time.
　　　　o = Observed clotting time in presence of ayapanin or ayapin.

The above formula enables one to get a quantitative idea regarding the extent of lowering of clotting time of blood and is independent of the nature of experimental animals, or the initial clotting time.

EXPERIMENTAL

Source of Blood.—Rabbits' blood, obtained by puncturing the ear-veins, was used in all the experiments. The animals weighed between 1020 and 1870 g. and belonged to both sexes. They were free from external injuries, and each animal was allowed a period of rest for at least one week after experiments, so that the wounds completely healed up, before they were used again.

Determination of Clotting Time of Blood.—The so-called lead-shot method was employed, in which highly polished and nickelled iron balls (weighing between 0.12 and 0.13 g.) were used. Two to three drops of freely-flowing blood were directly dropped on a clean, smooth (scratch-free) watch-glass in which a clean iron ball had been previously placed. The watch-glass was covered with a second watch-glass to prevent evaporation and then tilted up and down at a certain rate (usually 30 movements were

made per minute). Precautions were taken so that the warmth of the hand might not hasten coagulation. With the onset of coagulation the free movement of the iron ball was gradually retarded and finally arrested. The interval from the shedding of blood to the complete stoppage of the free movement of the ball was noted with the help of a stop-watch. This is admittedly the simplest of all known methods of determining clotting time of blood, but is not free from errors. But for our purpose which is concerned with the determination of relative clotting time, this method was found to be the most convenient and suitable.

Determination of Clotting Time in vitro.—A very fine film of the substance to be tested was deposited on the watch-glass along with the iron ball, blood was directly dropped on the vessel, and coagulation time determined by following the technique already described. Table I shows the results obtained.

TABLE I

Lowering of clotting time of blood in vitro *in presence of ayapanin and ayapin.*

Substance	Normal clotting time	Observed cotting time	H.A. (Hæmostatic activity
Ayapanin	135 secs.	70 secs.	48
	115	56	51
	118	60	49
Ayapin	120 secs.	60 secs.	50
	120	62	48
	118	57	51

It would thus appear that both ayapanin and ayapin hasten the clotting time of blood *in vitro* by nearly 50 per cent.

Experiments in vivo.—For this purpose we prepared solutions of ayapanin and ayapin in olive oil and sterilised them by heat. The normal clotting time of a healthy rabbit was then determined in the usual manner. A definite amount of ayapanin or ayapin (in oil) was then introduced into the animal by subcutaneous injection. Blood was then tapped out from different parts of the marginal ear-veins of the animal at different intervals ·of time, and the clotting time of the blood was determined in each case. Usually six to seven experiments could be performed on one animal, after which the animals could not be used further because the thrombokinase coming out of the injured tissues vitiated the results. In Tables II and III are recorded the results of the experiments.

TABLE II

Influence of ayapanin on the clotting time of rabbits' blood.

Substance in mg./kilo. body-weight	Normal clotting time (secs.)	Observed clotting time in secs. at different intervals* after injection							H.A.†
		5 min.	15 min.	30 min.	1 hr.	1½ hr.	2 hr.	2½ hr.	
0.3 mg.	200	170	163	173	165	—	—	—	18
	175	170	165	155	157	170	170	173	12
	127	122	122	112	109	103	118	—	19
0.5 mg.	112	—	92	80	100	105	115	—	29
	98	—	95	65	63	60	90	—	38
	115	—	95	77	100	93	98	—	33

TABLE III

Influence of ayapin on the clotting time of rabbits' blood.

Substance in mg./kilo. body-weight	Normal clotting time (secs.)	Observed clotting time in secs. at different intervals* after injection							H.A.†
		5 min.	15 min.	30 min.	1 hr.	1½ hr.	2 hr.	2½ hr.	
0.3 mg.	100	—	84	78	80	86	97	—	22
	96	—	75	72	78	86	98	—	25
	110	—	89	82	82	93	108	—	25
0.5 mg.	135	138	120	98	115	128	140	—	27
	130	135	120	94	115	140	128	—	28
	110	—	98	85	84	105	108	—	24

*The intervals refer to the period *after* injection.

†In evaluating the hæmostatic acitivity, much significance should not be attached to any figure of the order of 10 or less, because an experimental error of 10% may occur in the technique follwed by us.

The above data show that definite hæmostatic action of ayapanin and ayapin is noticeable with a minimum dose of 0.3 mg. per kilo body-weight approximately. A higher dose (0.5 mg.) enhances the hæmostatic activity, though not to the same extent in the two cases.

We next proceeded to observe the effects of the two compounds when introduced into rabbit *per os*. For this purpose olive oil containing definite amounts of the powdered substance was emulsified and put into the mouth of rabbits and the mixture washed down the throat with water. The clotting time of blood was then determined as before after regular intervals of time. The results are given in Tables IV and V.

TABLE IV

Influence of ayapanin given per os *on the clotting time of blood*

Substance in mg./kilo body-weight.	Normal clotting time (secs.)	Observed clotting time in secs. at different intervals after feeding.						H.A.
		15 min.	30 min.	1 hr.	1½ hr.	2 hr.	2½ hr.	
0.5 mg.	103	100	98	92	88	99	102	15
	130	122	115	110	118	124	126	15
1 mg.	125	82	80	100	105	118	—	36
2 mg.	90	—	57	—	60	80	92	37
	105	84	65	62	74	90	—	41
	125	103	90	86	80	96	85	36
4 mg.	113	65	60	80	70	70	—	47
	110	95	65	70	85	85	80	40
	108	76	72	60	85	90	—	44

TABLE V

Influence of ayapin given per os *on the clotting time of blood.*

Substance in mg./kilo body-weight.	Normal clotting time (secs.)	Observed clotting time in secs. at different intervals after feeding.						H.A.
		15 min.	30 min.	1 hr.	1½ hr.	2 hr.	2½ hr.	
0.5 mg.	98	95	90	82	84	92	96	16
	105	100	95	92	93	98	100	12
2 mg.	125	116	90	80	76	85	108	39
	108	105	91	76	64	68	100	41
	89	82	75	72	49	68	92	45

DISCUSSION

The results recorded in Tables II—V prove that both ayapanin and ayapin have the remarkable property of reducing the clotting time of blood when introduced into rabbit either by subcutaneous injection or by mouth. The substances have also been found to possess local hæmostatic action. The minimum dose necessary for definite hæmostatic action is slightly less than 0.3 mg. per kilo body-weight of the animal, when the substances are introduced by the subcutaneous route, whereas the corresponding dose *per os* is 0.5 mg. approximately. The activities of ayapanin and ayapin are practically of the same order. The maximum hæmostatic activity is believed to be of the order of 50, since experiments with doses of 20 and 40 mg. per kilo did not give figures above 50, although the duration of action was more prolonged. In the majority of cases the maximum lowering was observed between half and 1½ hours after administration. The normal value

of clotting time was restored practically in all cases in 2½ hours after administration, excepting when larger doses were given. In no instances did the animals show any outward symptoms of toxicity, even when extra heavy doses were given by mouth.* All the experimental animals continued to maintain health and growth. Consequently, it has not yet been possible to ascertain the M. L. D., which however must be considerably high in comparison with the minimum dose necessary to produce maximum hæmostatic effect. The therapeutic index therefore is high.

Some natural coumarins, particularly furo-coumarins, are known to be toxic towards fish and some insects, but the hæmostatic property of natural coumarins does not appear to have been discovered by any previous worker. Our observations consequently enhance the importance of natural coumarins.

The actual mechanism, which accelarates the clotting time of blood in presence of ayapanin and ayapin, has not yet been studied. The blood picture under the microscope however shows that there is no outward damage of the R. B. C. From the results reported in this paper it would appear that the use of *Eupatorium ayapana* in Ayurvedic system of medicine is perfectly justified. In view of the low toxicity of the compounds, the possibility of their use in clinical practice should be thoroughly explored.

We have also made some interesting observations regarding chemical constitution and hæmostatic action, and this would form the subject of the next communication.

SUMMARY

1. Ayapanin (7-methoxycoumarin) and ayapin (6:7-methylenedioxy-coumarin) are the active principles of *Eupatorium Ayapana*.

2. They are non-toxic and possess remarkable hæmostatic property. They are effective when applied locally, or when administered by subcutaneous injection or by mouth.

3. There is hardly any difference in activity among the two compounds.

4. The maximum hæmostatic activity does not exceed 50.

5. The mechanism of the physiological action of ayapin and ayapanin is not known.

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*On account of the low solubility of the substances in olive oil, subcutaneous injections with larger doses could not be conveniently given to the animals.

TOXICITY OF A DIALKYLAMINO ALKYLAMINO ACRIDINE AKIN TO 'ATEBRIN'

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In the course of the investigations on acridine derivatives as antimalarials (Basu and Das Gupta, 1) the dihydrochloride of 2-chloro-7-methoxy-5-(ω-diethylamino*iso*amyl) aminoacridine ('atebrin') was prepared and found to crystallise out from a mixture of alcohol and ether in microscopic yellow needles, m.p. 246-48°. It has now been described as "Mepacrine Hydrochloride" in the *British Pharmacopœia* (1932 Third Addendum, 1940, p. 14). As a dialkylaminoalkylamino acridine of this type is found (Kikuth, 2 ; Mauss and Mietzch, 3 ; Magidson and Grigorovski, 4) to possess a definite action on all asexual forms of the malarial parasites, the dihydrochloride of 2-chloro-7-methoxy-5-(δ-diethylaminobutyl)aminoacridine was also prepared by the condensation of δ-diethylaminobutylamine with 2:5-dichloro-7-methoxyacridine, and isolated as a yellow crystalline solid, m.p. 256-58°. An idea on the relative toxicity of the two products thus prepared, was considered to be of interest ; and the investigations so far carried out are being recorded in this paper.

5

<div align="center">EXPERIMENTAL</div>

Effect on Paramœcia.—*Paramœcium caudatum* was cultured on hay infusion (containing 0.5% glucose) maintained at pH ca.8. Equal parts of this culture and of the solution of the compound to be tested was taken in a chamber artificially made on a microscopic slide. The strength recorded in Table I is that of the above mixture which is, therefore, half that of the original strength of the acridine compound used. The period required for the death of the parasites as indicated by their becoming motionless and subsequent disintegration, was recorded. It has been noted that the pH of the 'butyl' and 'amyl' derivatives, in 1:1000 dilution was 5.8 and 5.75 respectively. With further dilution the pH varied from 6.05 to 6.1. Our culture of *paramœcium* was found unaltered in their motility even at pH 4.5.

<div align="center">TABLE I</div>

<div align="center">*Effect of the Acridine derivatives on Paramœcia.*</div>

Compound	Dilution	Period necessary for death
Dihydrochloride of butyl compound	1:1000	Immediate
	1:2000	,,
	1:4000	$1\frac{1}{2}$ minutes
	1:10000	$4\frac{1}{2}$,,
	1:20000	11 ,,
	1:40000	24-30 minutes
	1:80000	80 minutes (majority)
Dihydrochloride of amyl derivative: "Mepacrine Hydrochloride"	1:1000	$1\frac{1}{2}$ to 2 minutes
	1:2000	$1\frac{1}{2}$ to 2 ,,
	1:4000	2 ,,
	1:10000	9 ,,
	1:20000	35 ,,
	1:40000	75 ,, (majority)

Toxicity.—The toxicity was tested by subcutaneous administration of the drug on mice weighing between 20 and 22 g. each. Table II shows the results obtained by using the dihydrochloride of butyl and amyl derivatives. Deaths resulted from respiratory failure, some animals dying within half an hour.

TABLE II

Drug	Dose (mg./g.)	Mortality
Dihydrochloride of 2-chloro-7-methoxy-5-diethyl-aminobutylaminoacridine	0.175	4:20
	0.195	12:20
	0.200	14:20
Dihydrochloride of 2-chloro-7-methoxy-5-diethylamino*iso*amylaminoacridine or "Mepacrine Hydrochloride"	0.18	3:20
	0.20	7:20
	0.22	12:20
	0.24	16:20

The L.D.$_{50}$ was calculated by plotting the percentage mortality on a scale of normal equivalent deviations against the dose on a logarithmic scale according to the method of Gaddum (5). The L.D.$_{50}$ of the butyl derivative calculated in this way was 0.1897 mg./g., and that of the amyl derivative (Mepacrine hydrochloride) was 0.2116 mg./g. (Fig. 1) in mice.

FIG 1

Dose in mg /g (logarithmic scale)

'Butyl" Acridine

'Amyl" Acridine
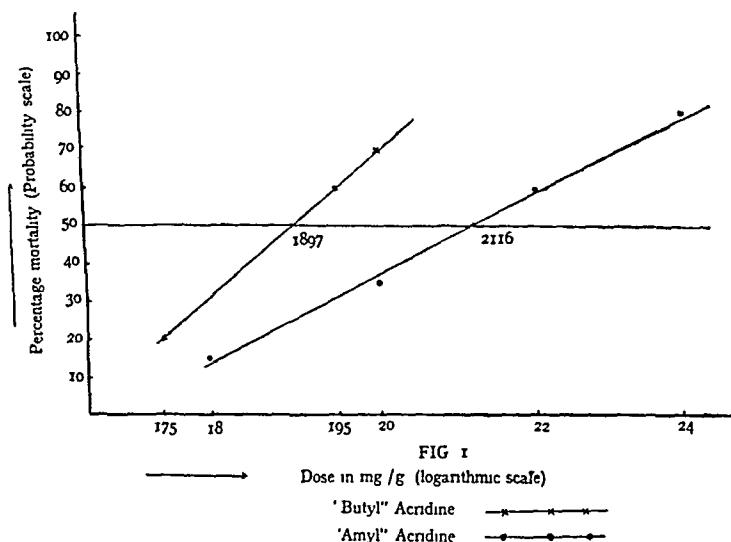
SUMMARY AND CONCLUSION

It may be noticed from Table I that the dihydrochloride of the butyl derivative is more toxic to paramœcia as the parasites were killed within four and half minutes when mixed even with a dilution 1:10,000. A similar dilution of the dihydrochloride of the amyl derivative (the active material of 'atebrin' tablet) causes death to the parasites in more than nine minutes. Moreover, the former is effective even in a still higher dilution.

This enhanced toxicity is also being manifested from its lethal dose in mice. The L.D$_{50}$ of the former compound is about 0.190 mg./g., whereas the corresponding figure for atebrin is found to be 0.212 mg./g. The respective slope of the curves in Fig. I indicates a uniform response of the animals used during the course of the investigation. It seems that a change in the characteristic *iso*amyl group present in the side-chain of the 'atebrin' (=mepacrine hydrochloride) increases the toxicity of the acridine derivative on the animals as well as on the protoplasmic organisms. The question remains whether such an enhanced toxicity is also associated with an increased antimalarial activity.

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THE ESTIMATION OF CYSTINE, TYROSINE AND TRYPTOPHANE
IN SOME COMMON EDIBLE FISHES OF BENGAL

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Science and Technology, Calcutta.*

The amino-acids, into which the proteins are resolved during the process
of digestion, pass unchanged into the circulation and thence they are distri-
buted throughout the organism. They are picked up by the living cells and
are utilized either for building up of tissue proteins or serve as the potential
source of energy. Thus the protein, or more correctly speaking, the amino-
acids, occupy a unique position in nutrition. Modern investigators have
revealed the presence of twenty-two naturally occurring amino-acids.
Strenuous researches have been made to determine the significance of the
individual amino-acids as dietary components and it has been proved that
some of these acids are indispensable for the maintenance of weight and
growth of the body and some are dispensable in the sense that they may be
synthesized in the body.

The amino-acids, cystine, tyrosine and tryptophane content of the
common edible fishes, a few of which are very often prescribed for the con-
valescents in Bengal have been determined, and the results are given in
Table I.

The unique rôle of tryptophane as a dietary component was very convincingly proved by the investigations of Osborne and Mendel (1). They observed that when a ration consisting of the protein, zein, whose tryptophane and lysine content is negligible is given to rats, they rapidly decline in weight and cease to grow. Inclusion in the zein diet of either of the above two amino-acids are needed for proper maintenance of weight and growth. This observation has recently been confirmed by Rose (2).

The early studies of Abderhalden (3) indicated that tyrosine is an essential nutritive and that phenylalanine, which has a very close similiarity in chemical configuration with tyrosine, may be mutually interchangeable in metabolism. But Womack and Rose (4) have advanced convincing evidences that it is phenylalanine which is the indispensable growth requisite and that it cannot be replaced by tyrosine.

Tyrosine, however, is believed to be the substrate from which adrenaline and thyroxine are synthesized in the body.

Cystine is of peculiar interest inasmuch as it is one of the very few sulphur containing amino-acids. The finding of Osborne and Mendel (5) was interpreted as demonstrating the essential nature of this acid. Recent investigations by Jackson and Block (6) have, however, disproved the indispensibility of this acid. They have shown that the sulphur-containing amino-acid, methionine, and not cystine, is really the indispensible amino-acid. Though methionine is essential for some vital functions, cystine can and does spare methionine for some other functions of the body. For example, in animals which normally produce large amounts of wool, hair, or feather, it would seem that dietary cystine might be of considerable advantage, since these structures contain large amounts of cystine and it would obviate the necessity of synthesizing cystine from methionine (7).

EXPERIMENTAL

Treatment of Fishes before Analysis.—Samples of fishes were completely dried in a vacuum oven at about 50°—70°. Before drying, the heads and visceras were thrown away and the protein material was made bone-free as far as was practicable. It took nearly 30 hours to drive away major portion of the water of the fishes. The dried samples were then defatted completely and were stored in air tight bottles in a cool place. Before actual analysis, samples were powdered in a mortar and put in a vacuum desiccator for two days until a constant weight was obtained. Definite quantities of fishes were also dehydrated at 110° to determine the total water content, and the ash was determined by incineration. The total nitrogen content of the fishes was determined by the micro-Kjeldahl method.

The method described by Folin and Marenzi (8) for determining the above amino-acids was adopted because of its relative reliability. The results of analyses are given in Table I.

TABLE I

Average of at least six determinations are given.

The figures indicate values in mg. per 100 g. of desiccated or fresh fish:

Bengali name	Zoological name	Desiccated and defatted fish				Fresh fish				Mtre	Ash
		CyN	TyN	TtN	Pr	CyN	TyN	TtN	Pr		
Magur	Clarius magur	134	282	116	98.21	23	48	20	16.95	80.91	1.86
Kai	Anabus scandens	148	317	97	94.55	22	46	14	14.64	81.46	1.06
Singi	Saccobranchus (Heteropneustes) fossilis	133	270	122	98.29	19	39	18	14.46	82.92	1.51
Lata	Ophiocephalus puctatus	86	264	92	96.38	12	38	13	14.47	80.62	1.67
Bata	Cirrhina reba	135	334	120	96.16	17	43	15	12.81	83.07	1.45
Bele	Gobius giuris	149	258	92	97.60	21	36	13	14.03	82.28	1.38
Bhangar	Mugil tade	118	268	114	98.33	16	38	16	14.03	80.45	1.63
Tangra	Macroneus vittatus	116	339	91	96.38	16	47	13	13.91	81.24	1.10
Puti	Barbus sarana	97	284	97	96.38	15	43	15	15.25	81.27	1.18

CyN = Cystine nitrogen.
TyN = Tyrosine nitrogen.
TtN = Tryptophane nitrogen.
Pr = Protein.
Mtre = Moisture.

SUMMARY

Cystine, tyrosine and tryptophane content in a few edible fishes of Bengal have been estimated. From the results obtained it is found that among the samples analysed, Singi (Saccobranchus fossilis) has the highest tryptophane content in the dried and defatted condition (122 mg.%) followed by Bata (Cirrhina reba) and Magur (Clarius magur), (120 mg.% and 116 mg.% respectively) whereas in the fresh condition, Magur (Clarius magur) contains the maximum amount of tryptophane (20 mg.%) followed by Singi (Saccobranchus fossilis), Bhangar (Mugil tade), Bata (Cirrhina reba). They

contain 18 mg.%, 16 mg.% and 15 mg.% respectively. Tangra (Macroneus vittatus) contains the maximum amount of tyrosine and is followed by Bata (Cirrhina reba) and Kai (Anabus scandens). In the fresh condition, Magur (Clarius magur), Tangra (Macroneus vittatus), Kai (Anabus scandens) and Puti (Babus sarana) contain almost equal amount of tyrosine. Bele (Gobius guiris) and Kai (Anabus scandens) contain the highest amount of cystine among the fishes investigated in the dry condition, whereas in the fresh condition these two fishes along with Magur (Clarius magur) contain the highest amount of cystine. Protein content in the fresh condition is highest in Magur (Clarius magur).

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A NOTE ON THE PREPARATION OF MUSTARD OIL RESEMBLING THE EPIDEMIOLOGICALLY INCRIMINATED SAMPLE IN PHYSICAL AND CHEMICAL PROPERTIES

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In a previous communication the present author (1) reported that it was possible to prepare a specimen of mustard oil from mustard seeds alone resembling in physical and chemical properties the potent mustard oil obtained from the field. The details of the method of preparation are now given.

While carrying out some investigations with pure mustard cakes an important observation was made . It was noted that cakes obtained from mustard seeds (argemone-free) after expression of oil in a bullock-driven 'ghani', when kept in a damp place, developed a kind of white mouldy growth on the surface and in course of time the whole mass was fully covered with this growth. The mould has provisionally been indentified to be a member of the *Moniliaceæ*.

It is well known that bullock-driven 'ghani' cakes are purchased in large quantities by some mill owners at a very cheap price and then reextracted in expeller mills to give about 6% mustard o'l. As the mustard cakes are very susceptible to fungal infection it was thought that the decomposition of these cakes or seeds may have some role in the production of toxic oil. This idea also received strong support from the observation of Goyal (2) that a fungus can be grown from the Rangpur oil and also from that of Dutt (3) and that a suspected mustard oil sample from Banares contained a large number of spores. It was desirable, therefore, to study whether the differential physical and chemical tests (4) would be applicable to the oil obtained from the fungus infected mustard cakes or seeds.

Sufficiently decomposed cakes were dried and powdered to a fine mass. The oil was then extracted with ether in a Soxhlet apparatus. The ethereal solution was treated with anhydrous sodium sulphate, filtered and the ether driven out. The extract so obtained was then heated in a steam-oven for a few hours. The mustard oil obtained in this way was fluorescent in the ultraviolet light and was strongly positive to the differential chemical tests for the epidemiologically incriminated oil, specially the nitric acid test, in which case the acid layer was almost blood-red. The solubility of this oil in alcohol was also much greater than that of pure mustard oil and in the

alcoholic solution it exhibited a broad absorption band between the regions 2912 A. U. and 2667 A. U. with the maxima near about 2747 A. U. The experiment with fungus infected mustard cakes was repeated more than once with practically the same result.

This finding suggests the possibility that a similar phenomenon may take place in mustard seeds during the rains specially if they are stocked in very damp places or if they get wet. An attempt was, therefore, made to develop this fungal growth on pure and dry mustard seeds by keeping them in an artificial humid condition and in contact with decomposed cakes. It took a fairly long time for the fungal growth to develop properly. The oil extracted as before by ether from these seeds gave a fairly positive nitric acid test. A portion of these infected seeds was then crushed slightly and kept in Roux culture tubes with sufficient water below. A copious growth was noticed within a few days and the oil obtained from this crushed mass by ether extraction was strongly positive to nitric acid test. From these experiments it is clear that at least by ether extraction of the decomposed mustard seeds and cakes it is possible to get samples of mustard oil which would respond to the nitric acid test very well. For the clarification of the problem, it is, however, necessary that expressed oil from the decomposed seeds should be positive at least to the nitric acid test.

With this end in view about 10 lbs. of mustard seeds (argemone-free) were thoroughly moistened with water and the mass then inoculated with a few pieces of infected cakes. After a short time the fungal growth could be noticed. The rate of growth was much accelerated by moistening the seeds with water periodically. When there was a copious growth then the seeds were expressed in a bullock-driven 'ghani'. *The oil thus obtained was distinctly positive to the nitric acid test.*

These investigations have, therefore, shown that it is possible to prepare samples of mustard oil from mustard seeds free from those of *Argemone mexicana* responding to the differential tests developed by Lal *et al* (4) for the epidemiologically incriminated mustard oil. If nitric acid test be the criterion of toxicity as has been assumed to be by Lal *et al* (5) then the mustard oil samples prepared here *may ultimately prove to be toxic.* Further biochemical investigations are in progress.

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Annals of Biochemistry and Experimental Medicine

Volume II, 1942

Edited by

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PHOSPHORUS CONTENTS OF THE SERUM, PLASMA -AND
WHOLE BLOOD OF TUBERCULAR AND DIABETIC
PATIENTS AND OF NORMAL PERSONS

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and Technology, Calcutta*

In various places investigations have been taken up to estimate the
calcium, magnesium and phosphorus contents of the blood in tubercular and
diabetic conditions (1). There is, however, much scope for regional investiga-
tions, particularly in Bengal, where very few data are available.

The metabolic complexities of these elements are reflected and repre-
sented to the highest degree in the blood and since it is a macrohomogeneous
material, blood has got to be considered with regard to its different com-
ponents, namely, corpuscles, plasma and serum.

Calcium is an essential component of all living cells. It appears to play an important part in decreasing the permeability of the cell membranes and the irritability of the cells in general. Phosphorus, through the formation of hexosephosphates, adenylic acid, creatine phosphate and co-carboxylase plays a primary rôle in carbohydrate metabolism, as well as in the fermentation processes. It is also concerned with the absorption of sugars from the intestine and the reabsorption of glucose in the kidney tubules. Through the formation of lecithin, it plays an important part in the lipid metabolism. In the intensive study of the behaviour and functions of calcium and phosphorus which has been in progress, there has been a tendency to minimise any possible rôle played by magnesium, the other bivalent cation of the body. Perhaps this has been partly due to the findings that magnesium, unlike calcium, does not vary constantly in any pathological condition, nor until very recently has it been found to be influenced by any experimental procedure. The striking effects of magnesium deprivation described by McCollum (2) and the findings of Greenberg and others (3), who have shown that after the injection of parathyroid extract, a rise in the serum magnesium occurs even earlier than the well-known rise in serum calcium, no longer justify us to overlook the importance of magnesium. The specific rôles played by magnesium in the life processes require special mention. It activates phosphatase and acts as a co-enzyme for the formation of hexosephosphates as a preliminary to carbohydrate breakdown in all living cells. It, therefore, is of fundamental importance for muscular work and yeast fermentation.

The total amount of calcium in the blood is confined to the plasma, there being none or only a trace, in the corpuscles. Greenberg and Mackay (4) and others have recorded that unlike calcium, more magnesium is present in the corpuscles than in the plasma. The greater amount of phosphorus is also confined to the corpuscles.

Disturbances of calcium, magnesium and phosphorus metabolism have been investigated in many pathological conditions. A preliminary investigation was taken up to determine these elements in the different components of the blood in samples obtained from normal, diabetic and tubercular Bengali males. The only evidence for an involvement of calcium and phosphorus metabolism in tuberculosis appears to be the calcification which is observed in tissues attacked by this disease. It is well-known that in diabetes a profound disturbance is caused to the general metabolism, particularly to carbohydrate metabolism. It will, therefore, be of interest to see to what extent magnesium and phosphorus constituents (which play a fundamental rôle in carbohydrate metabolism) are affected as a result of the disturbed metabolism.

It should be stated here that the pathological blood samples were obtained from correctly diagnosed untreated patients. The normal samples were drawn from healthy individuals.

EXPERIMENTAL

Magnesium has been determined according to the method of Greenberg *et al* (5) using 8-hydroxyquinoline. Phosphorus and calcium have been estimated according to the methods of Fiske and Subbarow (6) and Sobel *et al* (7) respectively. The results of analysis are given below.

TABLE I

Calcium, Magnesium, Inorganic Phosphorus and Total Acid Soluble Phosphorus in Whole Blood, Serum and Plasma of Normal Persons.

Figures express concentration in mg. per 100 cc.

Serial No.	Age	Ca/S	Mg/S	Mg/P	Mg/B	IP/S	TP/S	IP/P	TP/P	IP/B	TP/B
1	22	10.0	1.8			2.4	2.8				
2	41	11.4	2.2			2.6	3.0				
3	26	11.4	2.6			3.0	3.0				
4	29	9.6	3.2			2.8	3.4				
5	29	10.8	2.8			3.2	3.6				
6	33	10.8	2.8			2.6	2.6				
7	26	9.8	2.4			3.2	3.6				
8	20	11.2	2.2			3.0	3.4				
9	29	10.6	2.6			2.8	3.4				
10	31	9.8	2.2			2.6	3.2				
11	33			2.6	4.4			3.2	3.6	4.7	28.5
12	33			2.8	4.5			2.8	3.4	3.2	31.0
13	29			3.0	3.6			3.0	3.4	3.2	25.8
14	36			2.4	3.8			2.6	3.0	3.2	24.6
15	25			2.5	4.2			3.2	3.2	3.6	26.6
16	29			2.2	3.4			3.0	3.2	3.4	25.0
17	41			3.2	4.5			3.5	3.8	4.0	33.0
18	33			2.6	3.8			2.8	3.2	3.6	28.0
19	30			2.4	3.6			3.2	3.6	2.8	30.0
20	28			3.2	4.4			2.6	3.2	2.8	28.5

Ca/S—calcium in serum ; Mg/S—magnesium in serum ; Mg/P—magnesium in plasma. Mg/B—magnesium in whole blood ; IP—inorganic phosphorus ; TP—total acid soluble phosphorus.

TABLE II

Calcium, Magnesium, Inorganic Phosphorus and Total Acid Soluble Phosphorus in Whole Blood, Serum and Plasma of Tubercular Persons. Figures express concentration in mg. per 100 cc.

Serial No	Age	Ca/S	Mg/S	Mg/P	Mg/B	IP/S	TP/S	IP/P	TP/P	IP/B	TP/B
21	27	10.0	1.8			3.2	3.6				
22	23	10 2	2.2			2.8	3.2				
23	31	11.4	2.0			3.0	3.4				
24	25	9.6	1.6			3.4	4.2				
25	25	10 4	1.9			2.6	3.0				
26	26	10.4	1.9			2.6	3.0				
27	36	10.8	2.6			3.0	3.6				
28	19	11.2	2.8			2.4	3.2				
29	22	10.2	2.0			2.6	3.6				
30	22	10.8	1.8			3.2	3.8				
31	26			3.2	3.6			3.4	3.4	3.2	26.2
32	29			2.4	4.6			2.8	3.2	2.8	25.0
33	29			2.6	3.8			3.0	3.2	3.2	23.4
34	29			2.8	4.5			3.2	3.6	2.8	25.8
35	22			3.0	3.6			3.0	3.6	3.0	27.4
36	23			2.6	4.5			3.4	3.4	3.0	24.0
37	23			2.2	4.0			3.2	3.4	2.6	28 2
38	41			2.6	3.8			3.0	3.0	2.4	28.4
39	37			2.6	3.4			3.6	3.6	2.8	30.0
40	29			2.4	4.2			3.4	3.8	3.2	26.8

TABLE III

Calcium, Magnesium, Inorganic Phosphorus and Total Acid Soluble Phosphorus in Whole Blood, Serum and Plasma of Diabetic Persons. Figures express concentration in mg. per 100 cc.

Serial No	Age	Ca/S	Mg/S	Mg/P	Mg/B	IP/S	TP/S	IP/P	TP/P	IP/B	TP/B
41	34	9.8	1.8			3.0	3.2				
42	51	10.2	1.6			2.6	3.2				
43	54	10.6	2.2			3.2	3.8				
44	17	11.0	2.8			2.8	3.6				
45	36	9.6	2.2			3.4	4.2				
46	43	9.8	2.6			3.2	3.6				
47	51	10.4	2.8			2.6	3.4				
48	51	10.8	2.2			2.6	3.0				
49	47	10.4	2.6			2.4	3.8				
50	62	10.0	2.0			2.4	3.4				
51	61			2.8	4.4			3.4	3.6	2.8	28.0
52	59			2.6	4.2			3.6	3.6	3.2	30.5
53	54			3.0	3.8			3.0	3.4	3.6	34.0
54	27			2.4	4.2			2.8	3.5	4.0	32.0
55	39			3.2	4.0			3.0	3.6	3.6	24.6
56	43			3.0	3.4			2.6	3.0	3.4	26.0
57	47			2.6	4.2			3.5	3.8	3.8	25.5
58	47			2.8	4.0			2.8	3.2	3.6	28.0
59	59			3.0	3.6			3.2	3.2	3.4	27.8
60	57			3.2	3.4			3.4	3.6	4.2	26.5

The average mean values of the above findings are given in Table IV:

TABLE IV

Figures express concentration in mg. per 100 cc.

Condition of subject.		Ca/S	Mg/B	Mg/P	Mg/S	IP/B	TP/B	IP/P	TP/P	IP/S	TP/S
Normal	Range	9.6—11.4	3.4—4.5	2.2—3.2	1.8—3.2	2.8—4.7	24.6—33.0	2.6—3.5	3.0—3.8	2.4—3.2	2.6—3.6
	Mean	10.54	4.02	2.69	2.48	3.45	28.10	2.99	2.36	2.82	3.20
Tubercular	Range	9.6—11.4	3.4—4.6	2.2—3.2	1.6—2.8	2.4—3.2	23.4—30.0	2.8—3.6	3.0—3.8	2.4—3.4	3.0—4.2
	Mean	10.50	4.0	2.64	2.11	2.90	26.52	3.20	3.42	2.96	3.58
Diabetic	Range	9.6—11.0	3.4—4.4	2.4—3.2	1.6—2.8	2.8—4.2	24.6—34.0	2.6—3.6	3.0—3.38	2.4—3.4	3.0—4.2
	Mean	10.26	3.98	2.86	2.28	3.56	28.29	3.13	3.45	2.82	3.52

The distribution of calcium, magnesium and phosphorus in the blood of normal human subjects as determined by others is shown below.

TABLE V

Figures express concentration in mg. per 100 cc.

Author	Substance	Range	Mean
Watchorn and McCance (8)	Ca/S	9.0 — 11.4	10.3
	IP/S	3.0 — 5.8	4.0
	Mg/B	3.4 — 5.4	4.6
	Mg/P	2.0 — 3.6	2.7
	Mg/S	1.8 — 3.2	2.5
Greenberg (5)	Mg/P	2.44 — 3.04	2.74
	Mg/B	4.17 — 4.93	4.55
Kay and Byrom (9)	IP/B	2.8 — 3.4	3.1
	TP/B	24.3 — 30.6	26.6
Hamilton (10)	IP/S	2 — 5	
	TP/S	2.5 — 5.5	

DISCUSSION

It will be seen that serum calcium content in all the conditions is practically of the same order and that the values found are nearly similar to those of Watchorn and McCance (8).

The average magnesium content in the whole blood in diabetes and tuberculosis does not show any significant deviation from that of normal blood and the range is also similar. The maximum values obtained in all these conditions is 4.6 mg., whereas the values reported by Greenberg and others for normal blood range from 4.93 to 5.4 with a mean of 4.6 (Table V). The average value found in this investigation is 4.02. It appears, therefore, that the magnesium content of the whole blood of the Bengalis is somewhat lower than the values reported by Watchorn and McCance (8) and Greenberg (5).

The mean plasma-magnesium values of normal and tubercular persons are of the same order, with a slight increase in the diabetic condition ; whereas in the tubercular condition the serum-magnesium values are slightly lower than in the other conditions. It is to be noted, however, that the range of all the components in the different conditions studied are very nearly of the same order.

The inorganic phosphorus content of plasma and serum in tubercular condition is a little higher than in the other conditions, but the same constituent in the whole blood falls below normal in tuberculosis and it holds true in case of the total acid soluble phosphorus of the whole blood too. The total acid soluble phosphorus content in the normal whole blood is 28.10 (range, 24.6—33.0). The total acid soluble phosphorus of serum in diabetes and tuberculosis has been found to be slightly higher than normal, both in range and in the average mean value. Lastly the total acid soluble and inorganic phosphorus contents of the whole blood and serum in diabetes appear to be slightly higher than normal.

SUMMARY

The calcium, magnesium and phosphorus contents of normal, diabetic and tubercular Bengali males have been estimated in the different components of the blood. The following points are noteworthy:

1. The serum calcium and whole blood magnesium contents in the different conditions stated above are practically of the same order.

2. Magnesium content in serum and plasma can be considered to be equal to the values reported by Greenberg and others, whereas the whole blood magnesium is significantly lower than the values reported by the same authors.

3. The inorganic and total acid soluble phosphorus contents of whole blood in tubercular condition seem to be slightly lower than normal, but inorganic phosphorus, both in plasma and serum, is slightly higher.

4. The total acid soluble phosphorus of serum in both the pathological conditions has been found to be slightly higher than normal, both in range and in the average mean value.

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ROLE OF MANGANESE IN THE BIOLOGICAL SYNTHESIS OF ASCORBIC ACID: THE SYNTHESIS OF ASCORBIC ACID BY GUINEA-PIG INTESTINES

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In the previous communications of this series it has been shown by Rudra (1-4) that manganese plays an important rôle in the synthesis of ascorbic acid by plants as well as by animals. It has been found that seedlings obtained by steeping in solutions containing traces of manganese synthesise considerably larger amounts of ascorbic acid than the seedlings germinated in the absence of added manganese. Rat liver can synthesise ascorbic acid *in vitro* and *in vivo* in presence of sugars containing certain amounts of manganese. Guinea-pig liver can also similarly synthesise ascorbic acid from the sugar precursors in presence of manganese. But in this case the order of the amount of ascorbic acid synthesised is smaller than that synthesised by the rat tissue and moreover the concentration of manganese necessary is much higher than the concentrations required by the rat tissue. The small amount of ascorbic acid synthesised by guinea-pig liver *in vitro* and *in vivo* can be explained in part by the fact that the sugar used in the experiments are not the best precursors for the synthesis of ascorbic acid. Another explanation may be that the enzyme system in the guinea-pig liver has a weak activity. The author (6) has expressed the opinion that most of the ascorbic acid in animals is synthesised in part of the jejunal and ileac section of the small intestines. If this be true, then the experiments conducted with the small intestines, instead of with the liver, would be expected to give better results. Both *in vivo* and *in vitro* experiments have been performed with the small intestines of the guinea-pig in the following manner.

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EXPERIMENTAL

In Vivo *Experiments.*—Guinea-pigs of about equal size and reared upon the same diet were chosen for each set of experiment. They were divided into three groups. One group received only normal saline intraperitoneally. The second group received similarly the sugar precursor solution in distilled water and the third group received the sugar solution containing manganese. The animals were killed after five hours and a portion of the small intestines (jejunum), equally distant from the duodenum in all cases, was cut out and its ascorbic acid content determined. The results are given in Table I.

TABLE I

Ascorbic Acid Content of Guinea Pig Intestines after Various Injections

Expt. No.	Weight of animal.	Intraperitoneal injection given.	Ascorbic acid content of the intestines. mg./1 g.	Ascorbic acid synthesised mg./1 g. tissue.	
				Sugar alone given.	Sugar and Mn given.
I.	(a) 560 g.	1 cc. N.S.	0.22	—	—
	(b) 550	50 Mg. mannose in 1 cc. + 0.04% Mn (as MnCl₂).	0.30	—	0.08
II.	(a) 210	20 Mg. mannose in 0.5 cc. distilled water.	0.20	Nil	—
	(b) 180	20 Mg. mannose in 0.5 cc. + 0.04% Mn.	0.28	—	0.08
	(c) 188	0.5 cc. N.S.	0.20	—	—
III.	(a) 164	20 Mg. mannose in 0.5 cc. distilled water.	0.22	0.01	—
	(b) 160	0.5 cc. N.S.	0.21	—	—
	(c) 158	20 Mg. mannose in 0.5 cc. + 0.04% Mn.	0.30	—	0.09
IV.	(a) 155	0.5 cc. N.S.	0.36	—	—
	(b) 190	20 Mg. mannose in 0.5 cc. distilled water.	0.36	Nil	—
	(c) 170	20 Mg. mannose in 0.5 cc. + 0.04% Mn.	0.45	—	0.09
			Average	Nil	0.085

From the above results it is evident that when mannose solution in distilled water is injected into the guinea-pig, the same result is obtained as with normal saline ; no synthesis of ascorbic acid occurs whereas if the mannose solution is given in dilute manganese chloride solution then an appreciable amount of ascorbic acid (0.08 to 0.09 mg. per g. tissue) is synthesised.

In Vitro *Experiments.*—The *in vitro* experiments with the guinea-pig intestines were not very successful at first ; the incubated mixtures containing added manganese gave less ascorbic acid values than the controls containing no added manganese. This was probably due to the incomplete removal of food particles from the intestines, thus resulting in a high manganese concentration which becomes toxic to the enzyme system and also helps in the oxidation of the ascorbic acid already present. Some experiments were then performed with the intestines of starved animals and the results are given in Table II. More experiments of this type were not considered justifiable as another investigation of a more extended nature has been undertaken for proving the specific rôle of manganese in the synthesis of ascorbic acid by plants and animals.

TABLE II

Ascorbic Acid Content of Guinea Pig Intestines after Incubation for Three Hours in Ringer-Locke Solution

% Mn. in R.-L.	Water cc.	Mannose (20 mg.)	Ascorbic acid content of intestines (mg./1 g.)	Ascorbic acid synthesised (mg./1 g. intestines).	Remarks.
1. Nil.	1	—	0.19	—	
0.00005	—	1	0.23	0.04	
0.005	—	1	0.11	—	Ascorbic acid oxidised in a high conc. of Mn.
2. Nil.	1	—	0.13	—	
Nil.	—	1	0.13	nil	
0.00005	—	1	0.16	0.03	
0.0005	—	1	0.12	—	

The ascorbic acid contents were determined titrimetrically by the modified method of Birch, Harris and Ray (5). All the estimations were carried out at 0° and finished in equal time (about one minute). Under these conditions the reduction of the dye may be taken as equivalent to the ascorbic acid content of the material (*cf.* also Spruyt, 6).

DISCUSSION

The results obtained in the present experiments are considered as good evidence that in presence of a suitable concentration of manganese guinea-pig intestines can synthesise appreciable amounts of ascorbic acid from suitable sugar precursors (*cf.* also Milolo, 7, who considers that there is evidence for the synthesis of vitamin C by guinea-pig tissues). They also substantiate the opinion of the author (4) that the small intestines and not the liver is the probable seat of synthesis of ascorbic acid in animals. This is also in agreement with the opinion of Harde and Wolff (8) and of Hopkins (9) although Hopkins and Slater (10) later thought that both the small intestines and the liver are the seats of ascorbic acid synthesis in animals. It is also interesting to note that Wolfer and Hoebel (11) observed that there was a sharp fall in the ascorbic acid level of blood and urine in a case of jejunal feeding despite intravenous injection of the vitamin. This has been interpreted as due to the probable disturbance in jejunal function which interferes with the normal ascorbic acid metabolism of the patient. This appears to be an evidence in support of the author's theory of the jejunum being a probable seat of ascorbic acid synthesis. Widenbauer and Koschorrek (12) also found that slices of the small intestines, but not other tissues of the rat, could synthesise vitamin C.

Further evidence of the specific rôle of manganese in the synthesis of ascorbic acid by plants and animals has been obtained in this laboratory and the results will be communicated soon.

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NUTRITIVE VALUE OF SOME SPECIES OF THE SALT-WATER FISH OF THE WEST COAST OF INDIA

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Fish constitutes an important major item in the diet of a large section of the population of this country. This investigation comprises a systematic study of the amino-acid make up of the muscle proteins of three varieties of salt-water fish—Mullet (*Mulloides*), Karwa (*Lethrinus karwa*) and Ravas (*Polynemus tetractylus*) of the West coast of India. A knowledge of the amino-acid make-up of the proteins of fish is bound to be of importance for the nutritional worker in adjusting and correcting amino-acid deficiencies in foods by proper supplementation.

EXPERIMENTAL

Preparation of the Samples.—The head, tail and the possibly removable bones were rejected. After carefully skinning the fish, the edible muscle was stripped off from the bones, cut into thin slices and placed in a porcelain evaporating dish and dried in the steam oven. Since Remington and Shiver (1) and others have pointed out that there may be a variation among individuals within the same species, an effort was made to prepare representative samples by thoroughly mixing together the dried edible muscles from a number of fish of the same kind. The dry, powdered material was stored in glass-stoppered bottles in the cold room.

Analysis of the Proteins.—The proximate composition of the meals carried out according to the methods recommended by the Association of Official Agricultural Chemists is presented in Table I. The constituents of the ash are presented in Table II.

TABLE I

Composition of the meal expressed as percentages of the total material

Variety.	Moisture.	Ether extractives. (Fat)	Crude Protein. (N × 6.25)
Mullet	2.44	9.70	82.81
Karwa	5.62	6.12	80.76
Ravas	0.86	7.08	85.12

TABLE II

Ash constituents expressed as percentages of the fish meal

Variety.	Ash.	Calcium.	Phosphorus.
Mullet	5.96	0.069	0.117
Karwa	5.08	0.097	0.144
Ravas	6.92	0.158	0.143

Calcium was determined volumetrically after precipitation as the oxalate by permanganate titration and phosphorus by the method of Fiske and Subbarow (2).

The meal was defatted by exhaustive extraction with petroleum ether (b. p. 45°) and the fat-free residue (10 g.) was then thrice extracted with 300 ml. of 0.1% acetic acid each time on a boiling water-bath. The tissue was then digested for nine days at 37° with 0.1 g. of Pfanstiehl's pepsin in 200 ml. of 0.1 N-hydrochloric acid (3), filtered from any insoluble material, and the filtrate was made up to 300 ml. The filtrate contained 1200 mg. of nitrogen.

Aliquots of 100 ml. were hydrolysed with 25% hydrochloric acid at 120-125°, and the acid hydrolysates were analysed for the nitrogen distribution by the method of Van Slyke (4) with the following modifications:—

(1)   The amide nitrogen was determined by distillation in Parnas-Wagner apparatus for four and a half minutes.

(2)   The dicarboxylic-acid nitrogen was determined according to Damodaran (5).

(3)   The basic phosphotungstate was dissolved in sodium hydroxide and the phosphotungstic acid removed by means of barium chloride. This solution of the bases was used for the determination of arginine, (*a*) by boiling with 40% sodium hydroxide according to Van Slyke and (*b*) by the enzymic method of Hunter and Dauphinee (6), after the removal of amide nitrogen.

(4)   Histidine nitrogen was calculated in the usual way.

(5)   Independent determinations of tyrosine and tryptophan were carried out on 10 ml. aliquots of the peptic digest after alkaline hydrolysis by the colorimetric method of Folin and Marenzi (7).

(6)   Cystine was determined on further aliquots of 10 ml. of the peptic digest after acid hydrolysis by the method of Folin and Marenzi (8) as modified by Tompsett (9).   The results are presented in Tables III, IV and V.

TABLE III

Van Slyke nitrogen distribution of the muscle proteins
expressed as percentages of total nitrogen

Form of nitrogen.	Mullet.	Karwa.	Ravas.
Amide N	5.48	6.69	7.30
Humin N	3.52	1.45	1.11
Dicarboxylic acid N	18.90	19.72	26.84
Arginine N	19.77	23.75	14.31
Histidine N	11.20	3.58	13.43
Cystine N	1.56	0.86	1.46
Lysine N	5.21	8.31	2.64
Mono-amino N	31.94	36.30	31.58
Non-amino N	2.36	1.78	1.09

TABLE IV

Direct determination of arginine in solutions of hydrolysed muscle
proteins, expressed as percentages of total nitrogen

	Mullet.	Karwa.	Ravas.
Arginine N (Hunter and Dauphinee, 6)	21.02	23.26	15.86
Van Slyke	22.13	24.30	16.92

TABLE V

Independent determination of tyrosine, tryptophan and cystine on the hydrolysed peptic digests of the three species of fish

Amino-acid	Mullet		Karwa		Ravas	
	As % of total N	As % of protein (N × 6.25)	As % of total N	As % of protein (N × 6.25)	As % of total N	As % of protein (N × 6.25)
Tyrosine	22.38	3.58	18.3	3.05	14.80	2.37
Tryptophan	3.15	0.60	3.03	0.49	4.80	0.77
Cystine	13.32	2.13	7.33	1.25	12.60	2.02

CONCLUSIONS

From the above results, it is clear that the muscle protein of each variety of fish is appreciably rich in one or two essential amino-acids but markedly low in some others.

The arginine contents of both Mullet and Karwa (19.77 and 23.75%) are greater than that of Ravas (14.31%). Histidine content of Karwa is very low ; and that of Mullet and Ravas almost equal. Ravas contains less lysine than the other two. Both Mullet and Ravas contain higher percentages of cystine (2.13 and 2.02% respectively) than that of Karwa (1.25%). Both Mullet and Karwa are richer in tyrosine than Ravas, whereas the reverse is true for tryptophan.

The authors are indebted to Mr. B. N. Banerjee for his helpful advice and also to Dr. V. Subrahmanyan for his kind interest in the work.

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ON PLANT PHOSPHATASES. PART IV. THE 'OCCURRENCE OF "FREE" AND "BOUND" PHOSPHATASES IN SEEDS

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The study of the nature and properties of phosphatases present in resting seeds, as also their transformation during germination, has been the subject of many researches. Namec (1) found that the glycerophosphatase activity of plant seeds is greatly enhanced during germination. In experiments with barley and corn, he observed a lowering of activity in the first two days and then a great rise in the activity of the enzyme. Lüers and Malsh (2) also observed increase of phosphatase activity during the germination of barley. Ignatieff and Wasteneys (3) studied the presence and concentration of phosphatase in the different regions of plants (beans, potatoes, radishes and wheat) at successive stages of their life history, and found that in the germinated seed the enzyme concentration was much greater than in the dormant seed, and that the embryo of the ungerminated seed contained appreciable phosphatase activity, and only traces were found in the cotyledons. During the later stages of germination an appreciable quantity of phosphatase appeared in the cotyledons.

Very little information is available regarding the mechanism and nature of the phosphatase changes in seeds during germination. In previous publications Giri and Sreenivasan (4, 5, 6) have shown that the enzyme amylase occurs partly in an insoluble condition in rice, which cannot be extracted easily with water. During germination the insoluble enzyme is rendered soluble. It was, therefore, of interest to know whether a similar behaviour is exhibited by the enzyme phosphatase contained in seeds. The present investigation was undertaken with the object of obtaining information on (1) the occurrence of "free" and "bound" phosphatases in seeds, (2) the influence of germination on the activity and transformation of these

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enzymes, and (3) the influence of other factors like milling, parboiling of rice on the phosphatase activity.

EXPERIMENTAL

The seeds selected for the present study were rice (*Oryza sativa*), Ragi (*Eleusine coracana*), Soyabean (*Glycine hispida*) and green gram (*Phaseolus radiatus*).

Germination of the Seeds.—The seeds were first soaked in flowing tap water at room temperature for 24 hours, being afterwards transferred to trays containing a moist cloth, and allowed to germinate for varying lengths of time. In the case of rice, they were allowed to germinate in flowing water. After the appropriate period a portion was removed, dried under a fan and later in the sun, if necessary, and finally powdered. The powder was passed through a 40-mesh sieve, and preserved in bottles.

Determination of the Activity of the Phosphatase.—The activity was determined by estimating the amount of inorganic phosphorus released when a known amount of the active extract or powder was allowed to act on sodium β-glycerophosphate (Merck) in solution at pH 5.2 for a definite period of time at $35° \pm 0.2$ in an electrically controlled thermostat. Unless otherwise stated the reaction mixture contained 10 cc. of 2 per cent glycerophosphate solution, 10 cc. of M/5 acetic acid-acetate buffer (pH 5.2) and the enzyme material, the total volume being made up to 25 cc. The inorganic phosphorus was determined colorimetrically by the method of Fiske and Subbarow (7).

"Free" Phosphatase.—The phosphatase contained in the aqueous extract of the powder is designated as "free" phosphatase. This enzyme was obtained by extracting the powder with 10 times the quantity of water saturated with toluene in a refrigerator for 24 hours and filtering in a Buchner funnel.

"Bound" Phosphatase.—The phosphatase remaining in the powder after repeated extraction with water is designated as "bound" phosphatase. The activity of this enzyme was determined by allowing the powder, after repeated extraction with water, to act on the substrate and estimating the inorganic phosphorus released in the reaction mixture.

"Total" Phosphatase.—The phosphatase contained in the whole seed powder is designated as "total" phosphatase. The activity of the total phosphatase was determined by weighing a known amount (0.2 or 0.5 g.) of the powder and allowing it to act on the substrate, under exactly similar experimental conditions used for determining the activity of the "free" phosphatase.

"Free" and "Total" Phosphatases in Seeds

The activity of the "free" phosphatase was determined in an aliquot of the extract, which corresponds to 0.2 g. of the powder and the hydrolysis was allowed to proceed for 1 hour. In certain cases (marked with asterisk in the table) the volume of the aliquot taken for the determination of the activity corresponded to 0.5 g. of the powder. The activity of the total phosphatase was determined in 0.2 and 0.5 g. (in those cases marked with asterisk). The results are presented in Table I.

TABLE I

"Free" and "total" phosphatases of seeds

| | Phosphatase activity expressed in mg. P released in 10 cc. of reaction mixture. | |
	"Total"	"Free"
Rice (*Oryza sativa*) whole grain		
Sample 1.	0.295	0.070 (1st extraction)
		0.013 (2nd ,,)
		0.0 (3rd ,,)
Sample 2.	0.311	0.094
Sample 3.	0.207	0.060
Rice, hand pounded	0.222	0.050 (1st extraction)
		0.015 (2nd ,,)
Rice, polished	0.118	0.020
Ragi (*Eleusine coracana*)		
Sample 1.	0.068	0.025
Sample 2.	0.213*	0.081* (1st extraction)
		0.010* (2nd ,,)
Green gram (*Phaseolus radiatus*)	0.290	0.252
Soyabean (*Glycine hispida*)	1.560*	0.866* (1st extraction)
		0.252 (2nd ,,)
,, (defatted by treatment with acetone and ether)	1.230*	1.310*

It will be seen from the results that less than 50% of the phosphatase in rice and ragi exist in "free" condition which can be easily extracted with water, while the remaining part of the enzyme exists in "bound" form which cannot be extracted with water. In the case of soyabean, the phosphatase contained in the resting seed is extracted with difficulty and can only be obtained by repeated extraction, while the enzyme contained in the defatted material can be easily and completely extracted with water. Thus it is clear that the fat present in the seed interferes with the extraction of the enzyme. In the case of green gram, however, practically the whole of the phosphatase is present in "free" condition which can be completely extracted with water. It would seem, therefore, that the ease with which enzymes can be extracted

depends on the association of the enzyme with other substances present in the plant material. In the case of soyabean, it is probable that a part of the enzyme is associated with the fat contained in the resting seed thereby rendering the extraction of the phosphatase difficult. The amount of the "free" phosphatase present in rice is not affected by repeated extraction with water, and a large part of the phosphatase in rice is in "bound" form in the resting grain.

The influence of germination on the "bound", "free", and "total" phosphatase content of seeds.—The seeds were germinated in the usual way and samples were removed at stated intervals, and after removing traces of moisture, the seeds were dried either in a desiccator over P_2O_5 or under a fan. The dried seeds were then powdered and passed through a 40-mesh sieve. In Table II are presented the results concerning the activities of "free" and "total" phosphatases of seeds during germination.

TABLE II

The "free" and "total" phosphatase activities of seeds during germination

Germinating period.	Phosphatase activity expressed in mg. P released in 10 cc. of reaction mixture. "Free" phosphatase.				"Total" phosphatase.
	1st extraction.	2nd extraction.	3rd extraction.	Total.	
Rice (Oryza sativa) Specimen I.					
Resting grain	0.070	0.013	0	0.083	0.294
2 days (germinated)	0.208	0.016	0	0.224	0.254
3 ,, ,,	0.257	0.028	0	0.285	0.268
4 ,, ,,	0.284	0.055	0	0.339	0.325
Rice Specimen II.					
Resting grain	0.060	—	—	0.060	0.207
5 days (germinated)	0.070	—	—	0.070	0.160
Ragi (Eleusine coracana)					
Resting grain	0.081	0.010	—	0.091	0.231
1 day (germinated)	0.114	0.010	—	0.124	0.231
2 days ,,	0.450	0.100	—	0.550	0.627
3 ,, ,,	0.800	0.170	—	0.970	0.920
Soyabean (Glycine hispida)					
Resting seed	0.866	—	—	0.866	1.556
1 day (germinated)	0.932	—	—	0.932	1.510
2 days ,,	0.890	—	—	0.890	1.33
3 ,, ,,	3.60	—	—	3.60	3.50

It will be seen from the results that the activity of the "free" phosphatase of rice and ragi increases considerably during germination. The increase in the "total" phosphatase activity of rice is not very significant. In the case of ragi and soyabean. however, the "total" phosphatase activity increases on germination. Further it can be seen from the results that practically all the "soluble" phosphatase contained in the rice grain is obtained in the filtrate after the first extraction. It is clear from the results that the "bound" enzyme is transformed into "free" enzyme during germination.

The "bound" phosphatase in rice.—The activities of the phosphatase content of the powder (5 g. of the germinated and ungerminated rice) after repeated extraction with water were tested with a view to find whether the "bound" phosphatase is present in the powder in an active condition.

The digestion mixture, used for the determination of the activity of the phosphatase present in the residual material, consisted of 25 cc. of 2 per cent glycerophosphate solution, 50 cc. of M/5 acetic acid-acetate buffer of *p*H 5.2, 25 cc. water and the whole quantity of the residual powder after repeated extraction of the "free" phosphatase contained in 5 g. of the powder. The results are presented in Table III.

TABLE III

The "bound" phosphatase in rice during germination

Germinating period.				Activity of phosphatase expressed in mg. P released in 10 cc. of reaction mixture.
Resting grain	..	...	...	1.23
2 days germinated	...	...	...	0.71
3 ,, ,,	...	...	...	0.68
4 ,, ,,	...	...	...	0.05

The results show that the "bound" phosphatase content of rice diminishes gradually during germination.

The influence of milling and parboiling of rice on the phosphatase activity.—Giri and Sreenivasan (6) have shown that the enzymes amylase and phosphatase are mostly concentrated in the germ and the pericarp. The bran contains practically the whole of the phosphatase present in rice. The removal of bran and the germ would, therefore, considerably reduce the phosphatase content of the grain. In order to prove this, various samples of rice obtained from the market were examined and the results tabulated below clearly indicate the difference between the phosphatase content of hand-pounded and milled rice samples, the latter containing about 50 per cent. of the total phosphatase activity of the unmilled and hand-pounded samples.

Further, a sample of parboiled rice, which is prepared by steaming or boiling of unhusked rice after soaking in water, showed only very feeble activity of. the enzyme. This lowering of activity is probably due to the destruction of the enzyme by this treatment.

TABLE IV

The "total" phosphatase content of various samples of rice treated in different ways

Reaction mixture: 10 cc. acetate buffer M/5 (*p*H 5.2) ; 10 cc. 2% sodium β-glycerophosphate ; 0.2 g. of rice powder and 5 cc. water. Total volume, 25 cc.

No.	Treatment of rice.	Phosphatase activity expressed in mg. P in 10 cc. of reaction mixture.	
1.	*Whole grain* (including husk)		0.311
2.	,,		0.217
	Hand-pounded rice		
3.	,,		0.272
4.	,,		0.242
5.	,,		0.216
6.	,,		0.183
7.	,,		0.160
8.	,,		0.157
9.	,,		0.156
10.	,,		0.145
		Average	0.191
	Milled rice		
11.	,,		0.130
12.	,,		0.129
13.	,,		0.123
14.	,,		0.113
15.	,,		0.119
16.	,,		0.114
17.	,,		0.112
18.	,,		0.102
		Average	0.117
	Par-boiled rice		
19.	,,		0.015

DISCUSSION

The results obtained in the present study show clearly that

(*a*) The enzyme phosphatase occurs in certain seeds (*e.g.,* rice and ragi) in two different forms, namely as "free" phosphatase which is easily obtained by extraction with water and as "bound" phosphatase which cannot be extracted with water.

(b) During germination the "bound" phosphatase is rendered "free" and in the germinated seed the enzyme occurs mainly in "free" form.

(c) In certain seeds (like soyabean) the enzyme is partly associated with the fat and hence cannot be easily extracted with water.

Further characterisation of these two forms of the enzyme is being investigated. It would seem best, therefore, to look upon the increase in the phosphatase activity of the aqueous extracts of certain seeds during germination as being due partly to the physical alteration of the enzyme from the "insoluble", "bound" or "desmo" form into "soluble", "free", or "lyo" form.

Willstätter and Pollinger (8) have found a similar division of peroxidase into a soluble and insoluble fraction existing in horse-radish tissue. Similar types of enzymes which are physically different are found to exist in animal tissues (Willstätter and Rohdewald, 9, 10) and the terms, desmo- and lyo- enzymes have been introduced by Willstätter for characterisation of free and bound enzymes respectively (Willstätter and Rohdewald, 11). Using the nomenclature of Willstätter school, Bamann, Riedel and Diederichs (12) have shown that animal tissues contain lyo- and desmo-phosphatases.

The determination of the 'total" phosphatase content of various samples of hand-pounded and milled rices has shown that the phosphatase content of milled rices is on an average 50 per cent of that of hand-pounded samples. It would be useful to utilize this property for establishing milling standards based on the determination of the phosphatase content. It is necessary to study the relation between the phosphatase activity and vitamin B_1 content of rice milled to various degrees and to devise a "limit test" based on the determination of the phosphatase activity for assessing the nutritive value of rice with respect to vitamin B_1. Investigation in this direction is in progress.

SUMMARY

1. Phosphatases occur in two different forms (in certain seeds): one can be easily extracted with water and, therefore, exists in "free" form and the other, which cannot be extracted with water, therefore, exists in "bound" form associated with the insoluble material in the seed.

2. The "free" phosphatase content of rice and ragi increases and the "bound" phosphatase content decreases during germination. The increase in the "total" phosphatase content of rice during germination is very significant. In the case of other seeds there is a definite increase in the "total"

phosphatase content. During the germination of seeds the "bound" phosphatase is rendered "free".

3. The "total" phosphatase content of milled rice is on an average 50 per cent of that of hand-pounded samples.

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INVESTIGATIONS INTO INDIAN DIETS.   PART I.   THEIR EFFECT
ON THE HEALTH AND WELL-BEING OF THREE GENERATIONS
OF RATS*

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Dietary surveys in India have shown that most of the Indian diets are
ill-balanced and are deficient in calories, animal protein, animal fat, vitamins
and mineral salts, mainly those of calcium and phosphorus. McCay (1)
endeavoured to show that the difference in the health and physique of
various tribes in India was mainly to be explained on the difference in
their protein intake. McCarrison (2) extended these observations by
experimental studies on rats, groups of which were fed on diets similar to
those in common use by the people in different areas. He demonstrated a
striking parallelism between the health and physique of these animals and
those of different Indian tribes, the diets of which were fed to the rats.
In more recent studies Aykroyd and Krishnan (3) supplemented poor Indian
diets with other food-stuffs and concluded that milk was a better supplement
than soyabean and meat, for promoting growth and well-being of the ex-
perimental animals. They also obtained similar results in their feeding
experiments on groups of school children. These investigations, therefore,
throw a certain degree of doubt on the protein theory which McCay's earlier
work suggested. A critical examination of these defective diets, however,

*Part of the thesis for the Ph.D. degree of the Aberdeen University.

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indicates that they are grossly deficient in calcium and phosphorus, and hence the superiority of milk supplement over meat. This view is further strengthened by the fact that supplementing the diets even with pure salts of calcium and phosphorus results in great improvement in the health and well-being of the experimental animals (Aykroyd and Krishnan 4, Pal and Singh 5). In the preliminary study the effects of Indian diets on the growth, reproductive capacity and the degree of calcification of bones and teeth of rats were reported (Mullick and Irving 6). Observations have now been made on the value of Indian diets for maintaining health and well-being of a colony of albino rats. The studies include observations on growth, mortality, clinical condition and reproductive capacity of three generations of animals.

The Diets.—McCarrison's three typical Indian diets were selected for investigations (McCarrison 7), *viz.*,

(A) Northern Indian diet.

(B) Well-to-do Hindu family diet.

(C) The poor Hindu family diet.

The compositions of the diets as described by McCarrison are as follows:

(A) NORTHERN INDIAN DIET.

Atta	...	12.0 oz.
Rice (unpolished)		6.0 oz.
Dal	...	1.0 oz.
Meat	...	2.0 oz.
Meat	...	2.0 oz.
Oil	...	1.0 oz.
Ghee	...	1.5 oz.
Spinach	...	8.0 oz.
Veg. marrow	...	12.0 oz.
Milk	...	20.0 oz.

(B) WELL-TO-DO HINDU FAMILY DIET

Rice (polished)	...	23.0 oz.
Gram	...	1.9 oz.
Dal	...	1.2 oz.
Sugar	...	1.0 oz.
Cocoanut	...	2.0 oz.
Oil	...	1.2 oz.
Ghee	...	0.4 oz.
Spinach	...	3.0 oz.
Veg. marrow	...	3.0 oz.
Milk	...	7.0 oz.
Milk	...	7.0 oz.
Curd	...	9.0 oz.

(C) THE POOR HINDU FAMILY DIET

Rice (polished)	...	21.0 oz.
Dal	...	0.7 oz.
Gram	...	0.7 oz.
Oil	...	0.1 oz.
Cocoanut	...	0.05 oz.
Meat	...	0.09 oz.
Spinach	...	1.0 oz.
Veg. marrow	...	1.0 oz.

The analyses of these diets from different points of view are shown in Tables I, II and III.

TABLE I

Calcium and phosphorus content of the diets.

Diets	Ca	P	Ca/P
A	0.17%	0.35%	0.50
B	0.09	0.22	0.40
C	0.02	0.09	0.21

TABLE II

The distribution of the calories among the proximate principles of the diets.

Diets	Protein	Fat	Carbohydrate
A	13.0%	31.0%	56.0%
B	10.7	21.1	69.2
C	11.0	2.0	87.0

TABLE III

Diets	Vit. A (I.U.)	Carotene (I.U.)	Vit. B$_1$ (I.U.)	Vit B$_2$ (mg.)	Vit. B$_6$ (mg., rat unit)	Vit. C (mg.)
A	239	2370	418	0.236	75.0	53.0
B	145	785	68	0.420	91.0	15.7
C	0	415	59	0.470	121.0	8.1

The calcium and the phosphorus contents of the diets were determined in the laboratory from samples of mixed diets. It will be noted that all of them are low in calcium and especially diet C, which has the lowest Ca:P ratio. All the diets are poor in animal protein and animal fat. In diets B and C, an unduly high percentage of calories is derived from carbohydrates. The diets B and C are also poor in carotene and vitamins A, B_1 and C. In all the diets the quantities of calcium and phosphorus and the Ca:P ratio are so low that complete calcification is not expected to occur irrespective of the intake of vitamin D.

Albino rats bred at the All-India Institute of Hygiene and Public Health, Calcutta, for several generations were used throughout the experiment. The animals were divided into three groups of 48 animals each. Each group consisted of 24 males and 24 females 30 days old and approximately of equal weight and was fed one of the diets A, B or C. Half the numbers of each group (12 males and 12 females) were retained for breeding and for continuation of the study into the second and third generations. Of the rest, 8 animals (4 males and 4 females) of each group were killed at the age of 60, 90 and 120 days each time for the examination of their bones and teeth. Litter mates were so arranged in each group, that strictly comparable results could be obtained. Records of bi-weekly weighings were kept and standard methods employed for breeding. The purpose of continuing the study into three generations was to observe the possible remote and cumulative effects of the dietary.

EXPERIMENTAL RESULTS

Rate of growth.—The average growth rate of the animals in the different groups and different generations is summarized in Table IV. The animals of groups B and C failed to reproduce, hence in these groups observations in the first generation only were recorded. In group A, the litters were left with their mothers and ate the same food. They were weaned at the age of 30 days when the individual weight was about 25 g. The growth rate of the animals of group A was regular and steady whereas that of the animals of groups B and C was not at all appreciable in the first few weeks. After this period the animals of groups B and C began to put some weight but never attained the same weight as that of the animals of group A. The difference in the growth rates of the three groups in the first generation is clear from Table IV. The animals of group A had decreasing growth rate in the second and third generations.

TABLE IV

The average weights and growth rates of the animals.

Diet	Average wt. at birth in g.	Average weight in g. at ages in days.				Rate of growth per day (from 30 to 120 days) in g.	
		30		120			
		M	F	M	F	M	F

First generation

Diet	Average wt. at birth	M (30)	F (30)	M (120)	F (120)	M rate	F rate
A		25.0	23.8	82.6	80.7	0.63	0.60
B		28.2	26.9	46.7	45.2	0.20	0.19
C		26.5	24.3	38.7		0.10	

Second generation

A	4 5	24.4	23.7	77.2	76.1	0.57	0.55
B	*	—	—	—	—	—	—
C	*	—	—	—	—	—	—

Third generation

| A | 4.1 | 24.0 | 23.2 | 72.8 | 71.4 | 0.51 | 0.49 |

Indicates no breeding.

Mortality.—In group A, the death rate was 5% in the first generation and none in the second and third generations. In group B the death rate in the first generation was 10%. While in group C, the mortality was very high in comparison with other two groups. The rats, which died in this group, appeared very ill and lost weight very rapidly. In some cases the animals were so weak that they died while being weighed though they were handled with care. The cause of death in groups A and B was generally pneumonia and diarrhœa.

Clinical condition.—During the first three months of their life the animals of group A had a normal appearance. A large number of the animals in group C and a few in group B looked anæmic and their coats were rough. They were limp when handled and not at all playful and often vicious.

At the age of about 60 days, in the majority of cases the illness ran an acute course and ended fatally. During this period black rings appeared in the tails of the animals.

During the age of 90—120 days in group B the fur of the animals became smooth. Hardly any rat of group C lived beyond 90 days. Death in group C occurred as a result of diarrhœa, pneumonia and anæmia. The poor diet seemed to increase the susceptibility to various infections.

Reproductive capacity.—The animals of groups B and C were in too poor a state of health to be able to reproduce. In group B, the males and the females were kept together for 60 days from the age of 120—180 days without any pregnancy occurring. The animals of group C did not survive long enough to be mated. Thus animals of group A only reproduced. The observation of the reproductive capacity of the animals of group A are summarized in Table V.

TABLE V

Reproductive capacity of group A rats.

	Generations	
	2nd	3rd
Average time between introduction of males and birth of litters	34 days	48 days
Average number born per litter ...	7.4	6.9
Average number of still births ...	2.0	2.0
Average weight at birth	4.5 g.	4.1 g.
Average weight at weaning (30 days) ...	26.4 g.	24.6 g.

DISCUSSION

The average weight of the youngs in this study, taken six hours after birth, was 4.5 g. in the litters of group A, whereas the corresponding figure observed by Gaunt, Irving and Thomson (8) was 5.3 g. on their poor diet, which has the same Ca:P ratio as that of diet A. The average weight of the males of group A at 21 days was 17.0 g. This weight was too low

compared to those published by other breeders such as Donaldson (9), Simmonds (10), Mapson (11), Kellerman (12), Hain (13), Thomson (14) and Gaunt, Irving and Thomson (8). In all the cases the weight at weaning were much higher than those of animals taking our best diet. The rate of growth was also very slow in all the, three groups when compared with the figures of other workers. Gaunt, Irving and Thomson (8) showed that the rate of increase in weight per rat per day was about 1.0 g. on their diet which is comparable in composition with our diet A. In our animals during adult life the increase in weight of all the three groups was steady and regular though the weights attained at any age were always lower than those of the stock animals. During the whole course of the experiment, the death rate of the animals of both groups B and C was highest between 60 and 90 days, which is the period when rats usually have maximum growth and attain puberty. The cause of death was usually pneumonia and diarrhœa. The conditions of housing and of feeding of the animals of all three groups were the same, so that chances of infection were equal. The fatalities however occurred wholly among these rats and not in the animals on the stock diet.

So far as reproductive capacity is concerned our rats gave very poor results. From the results of other workers especially those of Cox and Imboden (15) one is apt to conclude that the Ca:P ratio in the diet is responsible for the poor results obtained. According to Cox and Imboden (15) the Ca:P ratio of the diet should be 1.0 to get the standard results of the reproduction. The Ca:P ratios in all our diets were below unity. The results obtained in this investigation suggest that 0.5 is the lowest limit of Ca:P ratio at which the rats will breed at all. The animals on diet C containing ricketogenic Ca:P ratio, with very low Ca and P content did not however produce clinical signs of rickets.

Summary

For two years a large colony of rats from the same stock was maintained on three typical Indian diets commonly used in India. An attempt was made to study three generations of these animals with regard to their growth, state of health, mortality and reproductive capacity. The differences in the nutritive value of these diets have been brought out in a striking manner ; even the best of these diets showed results very much poor in comparison to those obtained with standard diets.

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INVESTIGATIONS INTO INDIAN DIETS. PART II. THEIR EFFECT ON THE CHEMICAL COMPOSITION OF BONE AND TEETH*

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The effect of three typical Indian diets on the growth, well-being, mortality and reproductive capacity of a colony of rats maintained on the diets for three generations has been reported in Part I. In the present investigation the chemical compositions of the bones and teeth of the experimental animals are reported.

EXPERIMENTAL

Analysis of the ash of bones and teeth.—Observations are limited to the total ash, calcium and phosphorus content of the three bones (femur, tibia and fibula) of both the legs and the lower incisors. The tissues are cut from the bodies and all the adjoining muscles and organic materials are removed as far as possible. They are then refluxed with alcohol on water bath for six hours with four changes of the solvent and latter extracted with warm ether in a Soxhlet for 12 hours. After drying they are ashed in

*Part of the thesis for the Ph.D. degree of the Aberdeen University.

a silica crucible first gently and then in an electric muffle at 600°C for six hours. The ash was weighed and dissolved in a known volume of dilute hydrochloric acid. Calcium and phosphorus were estimated in duplicate on aliquots of the ash solution. Calcium was estimated by a modified method of Clark and Collip (1) introduced at the Rowett Research Institute, Aberdeen (Duckworth and Mullick, unpublished data). The method of phosphorus estimation was a modification of that of Fisk and Subbarow (2), all the estimations being done in a Pulfrich photometer, instead of a colorimeter.

In each group eight animals were killed (4 males and 4 females) at the ages of 60, 90 and 120 days. The results of analysis of the bones and teeth of these animals are summarized in Table I. The animals of groups B and C† did not breed.and hence the results of only one generation are given. In the first generation the percentage of the ash of the bones of animals of group C was in every case much lower than the corresponding values of the animals of groups A and B. The values of group B were lower than those of group A. The figures for the total ash of teeth show very little difference between three groups. These results are quite in agreement with the previous observations of Mullick and Irving (3) and of Guant, Irving and Thomson (4). The data given in Table I show practically no variation in calcium and phosphorus and their ratios, both in bone and teeth. It would therefore appear that the study of the percentage content of the calcium and phosphorus of the ash of teeth or bones or the ash content of the teeth is of little value as both these remain fairly constant under different conditions. The ash content of the bones furnishes a more suitable method in assessment of the nutritional value of the diets.

DISCUSSION

Sherman and Booher (5), Witcher, Booher and Sherman (6), Mullick and Irving (3) and Guant, Irving and Thomson (4) varied the calcium intake of their experimental animals and determined the ash content at different intervals of time. They showed that the ash content increased with the increased calcium content of the food.

From Table I it would be seen that the total ash content of the bones varied greatly with the difference in the calcium and phosphorus content of the diets. The good Indian diet A, which gave the best results of the three, failed in the present series of experiments to attain the normal standard. The bone-ash method for the assay of the nutritional value of the different diets as regards calcium and phosphorus utilization is considered as the standard method of Sherman, Irving and others.

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† These refer to groups nourished on diets B and C (*vide* Part I).

TABLE I

Chemical analysis of bones and teeth (average of four rats)

Age in days	Sex	Group	1st generation Wt. of ash in mg.	Bone ash %	Ca%	P%	Ca/P	2nd generation Wt. of Bone ash in mg.	Bone ash %	Ca%	P%	Ca/P	3rd generation Wt. of ash in mg.	Bone ash %	Ca%	P%	Ca/P
60	M	A	110.0	43.8	38.5	17.5	2.19	90.5	41.2	37.7	17.2	2.19	86.4	43.8	38.4	17.3	2.22
		B	50.7	36.2	37.6	17.8	2.11	—	—	—	—	—	—	—	—	—	—
		C	42.2	32.4	36.3	17.8	2.04	—	—	—	—	—	—	—	—	—	—
	F	A	105.8	44.5	39.0	18.7	2.07	90.8	43.0	36.8	17.9	2.05	80.5	45.0	38.4	17.5	2.19
		B	52.5	38.4	38.9	18.0	2.16	—	—	—	—	—	—	—	—	—	—
		C	41.7	34.3	36.5	18.8	1.94	—	—	—	—	—	—	—	—	—	—
90	M	A	150.2	46.2	38.9	17.8	2.18	128.2	46.5	38.2	18.2	2.10	109.2	43.9	37.8	17.3	2.18
		B	79.5	45.2	37.8	16.9	2.20	—	—	—	—	—	—	—	—	—	—
		C	44.1	31.8	37.3	17.3	2.16	—	—	—	—	—	—	—	—	—	—
	F	A	134.0	47.2	38.5	18.6	2.07	118.0	47.2	38.5	18.6	2.07	101.0	45.6	37.7	17.6	2.14
		B	67.6	45.8	38.0	17.4	2.18	—	—	—	—	—	—	—	—	—	—
		C	38.2	32.1	36.9	17.5	2.10	—	—	—	—	—	—	—	—	—	—
120	M	A	210.0	52.8	38.2	17.5	2.18	161.1	50.5	38.0	18.2	2.09	153.0	48.6	38.2	17.6	2.17
		B	91.6	46.8	37.66	17.9	1.10	—	—	—	—	—	—	—	—	—	—
		*C	45.8	31.0	37.6	17.9	1.10	—	—	—	—	—	—	—	—	—	—
	F	A	176.0	53.4	37.5	18.0	2.08	139.0	51.8	39.0	18.9	2.07	111.0	48.8	37.9	17.8	1.12
		B	77.0	47.5	38.0	17.3	2.20	—	—	—	—	—	—	—	—	—	—
		†C	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Teeth																	
60	M	A	27.6	68.5	41.7	18.8	2.22	26.2	68.0	40.6	19.1	2.12	28.2	69.5	40.8	19.4	2.10
		B	27.4	69.5	40.0	19.1	2.10	—	—	—	—	—	—	—	—	—	—
		C	22.2	66.0	40.00	18.8	2.12	—	—	—	—	—	—	—	—	—	—
	F	A	27.4	69.6	41.5	19.7	2.11	26.6	70.5	38.9	18.4	2.12	26.7	68.8	41.0	19.2	2.15
		B	26.4	69.0	41.5	19.5	2.12	—	—	—	—	—	—	—	—	—	—
		C	21.8	66.6	39.3	18.7	2.10	—	—	—	—	—	—	—	—	—	—
90	M	A	53.5	69.5	40.0	18.4	2.17	41.8	68.5	40.0	18.0	2.22	44.0	67.6	41.4	18.9	2.18
		B	47.5	69.0	40.1	19.4	2.06	—	—	—	—	—	—	—	—	—	—
		C	31.3	65.3	40.7	19.3	2.11	—	—	—	—	—	—	—	—	—	—
	F	A	48.6	69.0	41.5	19.9	2.09	37.8	69.5	38.8	19.5	1.99	41.4	67.5	41.2	19.1	2.16
		B	43.1	70.5	39.8	19.5	2.03	—	—	—	—	—	—	—	—	—	—
		C	26.8	66.0	39.8	19.7	2.00	—	—	—	—	—	—	—	—	—	—
120.	M	A	91.5	72.5	40.0	20.1	1.99	75.1	69.5	40.1	18.1	2.22	77.0	68.8	40.2	19.1	2.11
		B	71.5	69.4	41.2	19.5	2.12	—	—	—	—	—	—	—	—	—	—
		*C	40.4	66.4	39.4	19.8	1.99	—	—	—	—	—	—	—	—	—	—
	F	A	81.0	71.9	41.0	18.9	2.17	73.1	72.5	39.6	18.5	2.14	67.0	69.0	39.8	19.2	2.07
		B	65.0	70.4	41.1	19.8	2.08	—	—	—	—	—	—	—	—	—	—
		†C	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

*Average of two rats.　　†None survived.

SUMMARY

A large colony of rats all from the same stock were fed on three typical Indian diets for three generations and their effect on the chemical composition of the hard tissues was observed. The amount of the total ash of the bones of different diets containing different quantities of calcium and phosphorus varied greatly, whereas the amount of tooth ash and the calcium and phosphorus content of the ashes show very little difference in the three groups. The good Indian diet A which is the best of the three produced the greatest amount of calcification and the ash content of the bones of the animals on this diet was higher than that of the other two groups. However all the groups showed lower values when compared with those of a good stock diet. The bone ash method is shown to be a suitable one for the study of the nutritional value of the diets.

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INVESTIGATIONS INTO INDIAN DIETS. PART III. A HISTOLOGICAL TECHNIQUE FOR ASSAYING THE NUTRITIONAL VALUE OF THE DIETARIES*

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In Parts I and II the nutritional value of three typical Indian diets was studied as regards their effects on growth, general condition of health, mortality, reproductive capacity and the ash-content of bones and teeth.

Schour and Ham (1), Mullick and Irving (2) and Gaunt, Irving and Thomson (3) have shown that the technique based on the histological structure of teeth is much more sensitive than any other method for the study of calcification as a result of difference in calcium and phosphorus intake. In this investigation both X-ray and histological techniques were employed for the assay of the nutritional value of the diets. The investigation has been continued for two years into three generations of experimental animals, in order to observe the remote and cumulative effects of the dietary.

EXPERIMENTAL

(i) *Study of calcification by X-ray photographic method.*—In the initial stages of the experiment the estimation of calcification of the bones was made by X-ray photographs. The X-ray plates of litter mates of the same age of stock animals and those in groups A, B and C† showed varying degrees of bone calcification on different diets. Later examination of the histological structure of the teeth demonstrated more striking differences than those by the X-ray photographs. It was thought better, therefore, to study the calcification by the latter method only.

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*Part of the thesis for the Ph.D. degree of the Aberdeen University.

†These refer to groups of animals maintained on three typical diets, A, B and C (*vide* Part I).
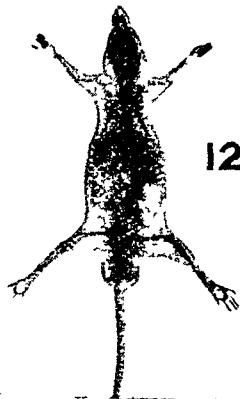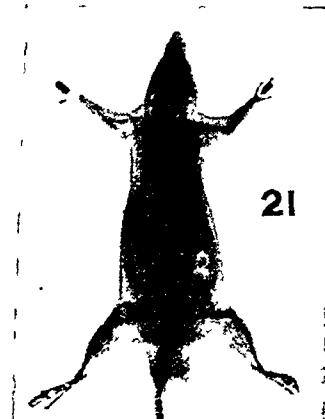
Stock   Group A
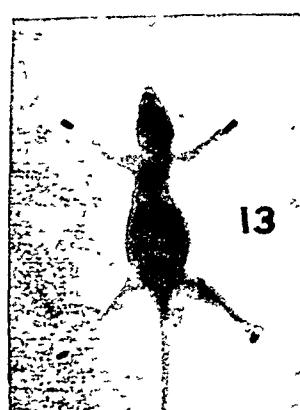
Group B   Group C

X-ray photographs of 90 days old male litter mates (first generation), showing the poor calcification in all the groups in comparison with the stock.

(ii) The histological method.—For histological examination, two upper incisors, the femur, tibia and fibula of the left leg were used, the histological examination of the bones failed to reveal any striking difference .among the three groups A, B and C and so it was given up. Histological examination therefore was confined only to the teeth.

The technique followed was those of Mullick and Irving (2) and Gaunt, Irving and Thomson (3). Longitudinal sections of the decalcified upper incisors were stained with hæmatoxylin and eosin and the predentine of the apical part of the tooth was measured. The normal value of the width of the predentine in case of animals receiving good stock-diet is from 16 to 20 μ Our experimental results in three generations of animals are given in the following table.

TABLE I

Width (μ) of the predentine in longitudinal sections of upper incisors of rats.

		1st generation Group			2nd generation Group	3rd generation Group
Age in days	Sex	A	B	C	A	A
60	M	36	39	44	39	39
	F	40	42	41	36	41
90	M	34	45	54	36	35
	F	38	41	51	35	38
120	M	36	44	56	37	38
	F	38	42	—	39	40

DISCUSSION

Typical photomicrographs of the stained sections of teeth of different groups are shown in Figures 1—4. These clearly demonstrate the differences in the effects of these diets. Schour and Ham (1), Mullick and Irving (2) and Gaunt, Irving and Thomson (3) have pointed out that the width of the predentine in the rodent incisor teeth offers a very delicate test for the degree of calcification. It becomes wider when calcification is impaired, the results in Table I demonstrate conclusively that calcification was deficient in the rats of all the three groups as compared to stock. The calcification of the group A rats improved with the age but the odontogenic line became wider in subsequent generations. The group B rats were worse and in some cases calcified globules were noticed in the dentine showing imperfect calcification. In group C, the width of the predentine increased gradually with age. The dentine formed was very poor and it was filled with calcified globules. The width of the predentine varied inversely as the ash content of the bones and directly as the calcium and phosphorus content of the diet.

McCollum's (4) technique of the line test was adopted by Steenbock and his co-workers (5, 6, 7, 8, 9 and 10), for routine tests connected with the control of the manufacture of irradiated foods and drugs under Steenbock's patent. The line test and the elaborate technique of Bill and Honeywell *et al* (11) have been adopted by the United States Pharmacopœia as the basis for vitamin D potency. A better and alternative method is the measurement of the width of the predentine (or odontogenic line of histological sections) for quantitative work on calcification. It may be suggested in this connection that histological examination of deciduous teeth will be valuable in assessing the previous nutritional history of children, especially with regard to Ca and P metabolism.

Fig. 1
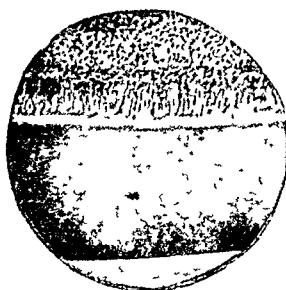
Stock

Fig. 2
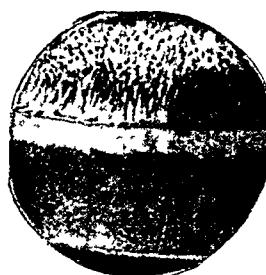
Group A

Fig. 3
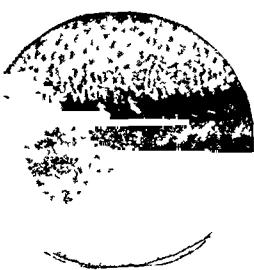
Group B

Fig. 4
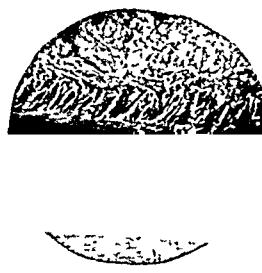
Group C

Longitudinal section of the apical region of the upper incisor teeth of ninety days old male litter mates (first generation) (× 200), showing poor calcification in all the groups in comparison with the stock.

Schour (12) has also employed a similar method for studying calcification but he used ground sections instead of ordinary decalcified sections. The latter give equally good results.

An advantage in studying the calcification from the structure of the teeth is that this hard tissue is mainly composed of calcium phosphate and the metabolic processes controlling their structure are in many ways similar to those influencing bone architecture. For purpose of study teeth have the advantage over bones that once formed (apart from absorptive processes leading to shedding of the deciduous teeth) their texture does not change like that of bones, so that a careful microscopic examination of fully developed teeth gives a much truer history of the metabolic changes to which they have been subjected during the developmental period, than is given by examination of bones.

Even slight changes in the Ca and P in the food bring about striking deviation from the normal appearance of dentine and predentine before any change can be noticed in X-ray pictures of bones.

It is remarkable that the population does not do so badly on these diets, especially diet C in comparison with the results obtained in the experimental animals. Calcium and phosphorus are probably the chief deficiency in these diets and this can be remedied by the use of milk and green foods.

SUMMARY

A large colony of rats was maintained on three types of diets commonly used in India. Three generations of animals were reared from the same stock on each of these diets. The rats on the "good Indian diet" (group A) gave the best results of the three.

Histological study of teeth is advocated as a test for the degree of calcification. The odontogenic line can be suitably utilised as the standard method for the evaluation of calcium assimilation of the experimental animals much as is the "line test" of McCollum and Steenbock.

I am also grateful to the authorities of the All-India Institute of Hygiene and Public Health, Calcutta and also to Prof. G. Sankaran for facilities and help offered for continuing the work at this Institute.

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INVESTIGATIONS INTO INDIAN DIETS. PART IV. A NEW
METHOD FOR THE STUDY OF CALCIFICATION

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In earlier papers of this series the nutritional values of three typical
Indian diets were studied with regards to their effect on growth, general
condition of health, mortality, reproductive capacity, ash content and
histological appearance of bones and teeth.

In this paper studies on the histological structure of the decalcified
sections of the teeth by the method of microincineration are reported.

Horning (1) studied the distribution of the inorganic element of cancerous tissues by this method. In the present investigation the method of micro-incineration of Horning with necessary modifications was adopted to study the calcification produced by different Indian diets.

The arrangement of the experiment is the same as reported in Part I. The study was confined to first generation of rats nourished on the three typical diets.

RESULTS AND DISCUSSION

With the help of this method one can locate the cytological distribution of the mineral substances and can study the distribution and the disturbances of the inorganic salts especially calcium both in the normal and pathological state of the teeth. The photomicrographs (Plates 1-3) clearly demonstrate the difference in the degree of calcification. The white area represents the predentine or odontozonic zone of ordinary sections and the distribution of the white spots clearly shows the disturbances in the normal formation of the dentine. It also appears that the white area varies directly with the nutritional value of the diets. We find that the microincineration method is valuable for determining the degree of calcification. In fact this method seems to be better than the ordinary histological method of studying the predentine of the defective teeth. If the photomicrographs of the incinerated sections are compared with the ordinary histological sections, one is impressed with the ease with which defective calcification as shown by the width of the predentine and globules in dentine can be judged by the former method. The ash of the incinerated sections should contain all the mineral constituents which are not volatilised at 650°. The suggestion that the distribution of salts in incinerated preparations resembles their distribution in living cells is based on the observation of Scott (2). In the succeeding sections of the above paper experimental results suggest that the inorganic constituents of the cell retain an unexpected constancy in *relative positions* during the process of micro-incineration and that if any error occurs it is only a slight one. The Ca ions in combination with protein are in a very stable condition and cannot be removed even with strong acids or alkalies. A study of the photomicrographs of these incinerated sections will demonstrate the ease with which slight disturbances in the calcification of teeth can be clearly made out. The white area represents the mineral residue of the cellular tissue observed in the dark ground illumination. While the histological staining method shows in cases of deficient calcification striking

changes in morphology, micro-incineration technique goes further in making as it were a micro-chemical analysis of cells in situ. Careful examinations of large number of these sections have brought forth the striking fact that

PHOTOMICROGRAPHS OF RAT'S INCINERATED INCISORS BY DARK GROUND ILLUMINATION

| Group A | Group B | Group C |
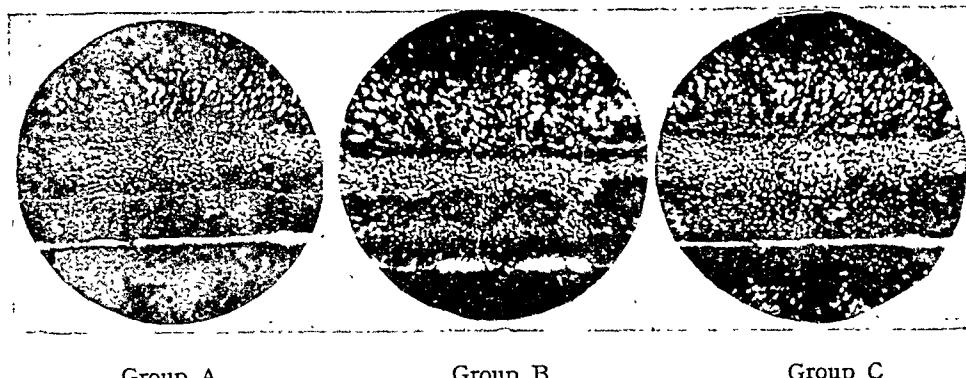
Longitudinal incinerated sections of the apical region of the upper incisor teeth of ninety-day male litter mates (× 200), first generation.

Note.—The white area and the white spots of the incinerated sections vary with the nutritional value of the diets.

faulty mineralisation of the teeth is not a matter of poor or inadequate ingestion alone but is due to one or more of the following factors:—

(*a*) Impaired function of the cells at the dental area. For, how else can one explain the heavy mineralisation of the widened predentine rendered evident by the new technique. One gets the impression here that the transference of minerals by odontoblasts to the dentine is interfered with.

(*b*) The pathological condition of the predentine. In a faulty diet the normal mechanism of calcium and phosphorus assimilation is disturbed and thus there is also disturbance of these elements in colloidal combination in the circulating fluids and thus instead of formation of dentine by the cells there is wider appearance of predentine and calcified globules.

(*c*) The increased predentine shade in micro-incinerated sections of the teeth showing deficient calcification may be due to the minerals being drained out of the dental area towards dental pulp.

Whatever may be the reasons of faulty mineralisation of teeth, it is best studied by means of this new technique of micro-incineration.

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COMPLEMENT FIXATION TEST IN KALA-AZAR

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The work on complement-fixation in Kala-Azar has been concerned with both specific and non-specific test antigens. Earlier work yielded discordant and mostly negative results. Heavily infected human spleen, dead protozoa from NNN culture media or spleen from immunized rabbits, were used for the preparation of antigen. Later, however, Hindle, Hou and Patton (1) used an infected hamster spleen and prepared antigen by triturating in normal saline, straining through muslin and adding 1% Glycerine and 0.5% phenol. They obtained positive result in all the cases of Kala-Azar diagnosed by Liver puncture, but at the same time 11% of non-Kala-Azar sera also showed fixation of the complement. Auricchio (2) used 10 to 12-day cultures suspended in normal saline and obtained 80% positive results. Georgiewsky (3) obtained specific fixation with alcoholic extract of dried Liver from cases of human Kala-Azar, but the antigen would also give positive results with malarial and syphilitic sera as well. Da Cunha and Dias (7) used alcoholic extracts of acetone washed Leptomonads from blood-agar plates and obtained very good results with the antigen. Nattan-Larrier and Grimard-Richard (5) used hyper-immunized rabbits' serum prepared by the injection of Leptomonad form of L. donovani in fixing the complement with Kala-Azar serum. But Anderson and Disdier (6) have not found this test sufficiently specific to be of diagnostic value. Da Cunha (4) used an antigen prepared from Trypanosoma Equiperdum and obtained positive results only with chronic cases of L. donovani infection and negative results with comparatively acute cases. Greval, Sen-Gupta and Napier (8) used Witebsky-Klingenstein-Kuhn antigen (W.K.K.) and found that out of 132 cases not a single diagnosed case of Kala-Azar gave a negative reaction. But this antigen, as can be expected, reacted positively with sera from cases of Leprosy and Tuberculosis.

PRESENT INVESTIGATION
Technique

Antigen :

A 48 hours' growth of *Leishmania donovani* in Ray's media at room temperature was washed with normal saline. In another tube the growth was washed with distilled water. Both the tubes were brought to a strength of 60 million organisms per c.c. The suspensions were then put into a shaking machine for 48 hours, at the end of which the antigens were preserved in a refrigerator. The distilled water antigen was made isotonic with the addition of sodium chloride up to 0.85% before final preservation.

Serum :

Blood was collected from the patients in the morning in empty stomach or in the afternoon 4 to 5 hours after midday meal. The serum was separated after being kept in the incubator for 1 hour and in the refrigerator overnight. This was inactivated for $\frac{1}{2}$ hour at 56°C before the test and 0.1 c.c. was used for the test as well as for the serum control.

Complement :

Pooled guinea-pig serum was used.

R.B.C. and Hæmolysin :

R.B.C. from sheep's blood collected on the same day and hæmolysin produced in rabbits against sheep R.B.C. were used. For the test 5% R.B.C. suspension was used.

Hæmolytic System :

For the final test as well as for the titration of the antigens, 2 units of complement and 2 units of hæmolysin were used.

Titration of Antigens :

1. Hæmolytic Unit:

Undiluted antigen	...	...	0.1 cc.
Normal Saline	...	...	1.7 cc.

incubated at 37°C for 1 hour

R.B.C.	...	...	0.2 cc.

incubated at 37°C for $\frac{1}{2}$ hour

Both the antigens were tested and no hæmolysis was observed to occur.

2. Anti-complimentary Unit:

Antigen	— Dilutions 1:	1	2	4	8	16	32
	Volume used	0.1 cc.	0.1 cc.	0.1 cc.	0.1 cc.	0.1 cc.	0.1 cc.
Complement	— 2 units·						
	volume	0.1	0.1	0.1	0.1	0.1	0.1
Normal saline		1.5	1.5	1.5	1.5	1.5	1.5

incubated at 37°C for 1 hour

R.B.C. (5% suspension)		0.2 cc.	0.2 cc.	0.2 cc.	0.2 cc.	0.2 cc.	0.2 cc.
Hæmolysin	— 2 units·						
	volume	0.1	0.1	0.1	0.1	0.1	0.1

incubated at 37°C for 10 mins.

Result :

Dilutions 1 :	1	2	4	8	16	32
Normal saline antigen	H	H	H	H	H	H
Distilled water antigen	NH	H	H	H	H	H

H = Hæmolysis and NH = No-Hæmolysis.

THE TEST

Dilutions of antigens used are 1:2 of normal saline antigen and 1:8 of distilled water antigen.

(i) *An ensemble :*

Materials	Test Proper	Serum control	Antigen control	System control	R.B.C. control.
Normal Saline	1.4 cc,	1.5 cc.	1.5 cc.	1.6 cc.	1.8 cc.
Serum	0.1 cc.	0.1 cc.	—	—	—
Antigen	0.1 cc.	—	0,1 cc.	—	—
Complement (2 m.h.d.)	0.1 cc.	0.1 cc.	0.1 cc.	0.1 cc.	—
	— kept in the Incubator at 37°C for 1 hour				
R.B.C. (5 % Suspension)	0.2 cc.	0.2 cc.	0.2 cc.	0.2 cc.	0.2 cc.
Hæmolysin (2 m.h.d.)	0.1 cc.	0.1 cc.	0.1 cc.	0.1 cc.	—
	— kept in the Incubator at 37°C				

The results are read 10 mins. after complete hæmolysis in the control tubes.

(ii) *Reading of results :*

Complete inhibition of hæmolysis + + +
Trace of hæmolysis + +
Hæmolysis almost complete +
Complete hæmolysis −

(iii) *Result :*

TABLE

Cases :	Sera :	Aldehyde Test	Urea Stibamine Test	D. W. Antigen	Saline Antigen
Kala-Azar	8	Strongly positive	Strongly positive	+ + +	+ + +
	2	Negative	Weekly positive	+	+ + +
Dermal Leishmaniasis	2	Negative	Weekly positive	+ +	+
Normal	5	Negative	Negative	—	—
Malaria	3	Negative	Negative	—	—
Tuberculosis	2	Negative	Weekly positive	—	—
W. R. positive	3	Negative	Negative	—	—

7

REMARKS

For the Urea Stibamine Test the sera were diluted to 1 : 10. The Kala-Azar cases were diagnosed on clinical picture, history, results of Aldehyde and Chopra tests and by the effect of Antimony on them. Malaria cases were diagnosed by finding malaria parasites in blood and by the effect of Quinine, while Tuberculosis was detected in both the cases by finding T.B. in sputum. None of the Tuberculosis cases had enlarged Spleen or Liver nor did they show any symptom of Kala-Azar.

SUMMARY AND CONCLUSION

1. Two types of antigens were prepared for complement fixation with Kala-Azar sera.

2. The test was conducted with 10 Kala-Azar sera and with 15 other sera as control.

3. All the Kala-Azar sera gave positive reaction while all the other sera were negative.

4. It is suggested that further trial may perfect this test for diagnostic measure.

ACKNOWLEDGEMENT

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COMPLEMENT FIXATION OF HUMAN SERUM IN *PLASMODIUM VIVAX* INFECTION WITH *PLASMODIUM KNOWLESI* ANTIGEN

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Successful complement fixation reactions in human malarial infections have been reported by several workers (1—6). Thomson (2) got indication from preliminary work that a given test antigen showed a group reaction for all the species of human malaria. Kingsbury (5) also got the same result. Savtchenko and Baronoff (6) reported specific reaction with their alcoholic antigen. Coggeshall and Eaton (7) reported successful results in monkey malaria using homologous antigen. In a later paper (8) results obtained with *Plasmodium knowlesi* antigen and human sera during *Plasmodium Vivax* infection were recorded by the same authors. Recently, Ray, Mukherjee and Roy (9) reported positive reactions with the sera of monkeys immunised with *Plasmodium knowlesi* antigen.

Antigen :

The material used as antigen was prepared from spleen removed ·from a monkey during heavy infection with *Plasmodium knowlesi*. The spleen was finely chopped and dried in a vacuum desiccator at 37°C for two days and then powdered. 10 c.c. of fluid per 1 g. of dried material was used for extraction of the antigen. The pulv was extracted with

- (*i*) Normal saline at 4°C for 3 days.
- (*ii*) Normal saline at 37°C for 3 days with toluol as preservative.
- (*iii*) Distilled water at 4°C for 3 days.
- (*iv*) Distilled water at 37°C for 3 days with toluol as preservative.

   (*v*) Distilled water by alternate freezing, and thawing at 37°C for
          3 days.
  (*vi*) Normal saline at room temperature (21°C-13°C) with 0.5 per cent.
          phenol as preservative for 1½ years.

The extracts were passed through filter paper and the filtrates were used as
antigens, in suitable dilutions.   By titrating against a single positive serum
from a monkey having acute *Plasmodium knowlesi* infection, it was found
that the antigen No. (*vi*) was the strongest, while Nos. (*ii*) and (*iv*) in which
toluol was used as preservative were the weakest.   The antigens were titrated
for anti-complementary and hæmolytic properties.   No marked hæmolytic
activity was noticed with any of them while anti-complementary effects were
present only in 1:2 dilutions in antigens Nos. (*v*) and (*vi*) and in undiluted
conditions in Nos. (*i*), (*ii*), (*iii*) and (*iv*).   In the test, a dilution of antigen
which was 3 times the anti-complementary dilution, was used.

Sera :

   During acute infections three sera were obtained on the 3rd day of fever,
one on the 5th day and another on the 16th day.   None of them had quinine
before.   Among the chronic cases one serum was obtained during relapse
on the 2nd and three on the 3rd day of fever and four during afebrile periods,
15 days, 29 days, 30 days and 58 days after the last relapse.

Method of performing the Complement-Fixation test :

   Serum was used in 0.2 c.c. quantity, undiluted.   Guinea-pig serum was
used for complement which was titrated each day using 1 unit of amboceptor
and the hæmolytic system consisted of 5 per cent sheep R.B.C. and anti-sheep
R.B.C. serum.   The unit of amboceptor was the smallest amount which
produced complete hæmolysis in the presence of complement.   The actual
test was set up as follows:

Serum   ... 0.2 cc.
Complement   ... 0.1 cc.   (2 units).
Antigen No. vi   ... 0.2 cc.   diluted as stated before.
Normal saline   ... 1.0 cc.

   Controls used are
     1. Immune serum with normal saline 1.2 c.c. and complement.
     2. Antigen with normal saline 1.2 c.c. and complement.
     3. Complement and saline 1.4 c.c.
     4. 0.2 c.c. normal serum with complement and antigen and saline.
     5. 0.2 c.c. W. R. positive serum with complement and antigen and
         saline.
     6. Immune serum with normal spleen extract 0.2 c.c. with comple-
         ment and saline.

The normal monkey spleen was extracted as antigen No. (*i*). The tubes were incubated at 37°C for 1 hour and 0.3 c.c. of 5 per cent sheep R.B.C. and 0.1 c.c. amboceptor (2 units) were then added. The tubes were again incubated and readings made 15 minutes after complete hæmolysis in all the control tubes.

Result :

The results are recorded as strongly positive (+ + +), moderately posiive (+ +) and weakly positive (+). A rough idea of the number of malaria parasites present in smear from peripheral blood is also indicated in a similar way. All the control tubes recorded complete hæmolysis.

Acute Infection :

Serum	Day of fever	M. P.	Result
1	3	+	+
2	3	+ +	+ +
3	3	+	—
4	5	+ +	+
5	16	+ + +	+

The complement fixing antibody was present rather early in three of these sera and its amount did not run parallel to the number of infecting parasites in the peripheral blood.

Chronic Infection :

Serum	Day after relapse	Day of fever	M.P.	Quinine	Day after Quinine	Result
6	—	3	+ +	—	—	+ + +
7	—	2	+ +	20 grain	2	+ + +
8	—	3	+ +	10 grain	1	+ + +
10	29	—	—	60 grain	20	—
9	30	—	+	40 grain	2	+ +
11	15	—	+ +	—	—	+ +
12	58	—	+	60 grain	10	+

The largest amount of complement fixing antibody was present during relapse of the fever in cases 6, 7 and 8, while in sera collected in afebrile periods the reactions had been weaker. In serum No. 10, which was from the same patient as serum No. 6 and which was collected 20 days after a course of quinine resulting in complete cure, no complement fixing antibody could be detected.

CONCLUSION

1. *Plasmodium Vivax* infection in man gives rise to a complement fixing antibody reacting with *Plasmodium knowlesi* antigen.

2. The amount of complement fixing antibody does not run parallel to the number of malaria parasites present in the peripheral blood.

My best thanks are due to Dr. J. C. Ray for his interest and advice.

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EFFECT OF *PLASMODIUM KNOWLESI* ANTIGEN ON ACUTE AND CHRONIC INFECTIONS WITH THE HOMOLOGOUS STRAIN OF PARASITE IN *M. RHESUS*

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The present investigation was undertaken with a view to find the effect of an antigen containing dead *P. knowlesi* on acute and chronic infections with the same strain of parasite.

METHOD

Two groups of Rhesus monkeys were selected for experiments. Group I consisted of 2 monkeys suffering from chronic infection for the last 10 and 14 months. Group II consisted of another 2 normal monkeys which were infected during the course of the experiment. In both groups definite amount of *P. knowlesi* antigen were introduced intravenously, in group II only after the appearance of the malaria parasite in the peripheral circulation and the effects on the parasite rate and on blood count, were noted.

EXPERIMENT AND RESULT

Preparation of the antigen—Monkeys were infected with *P. knowlesi* and bled to death when the peripheral circulation showed a very heavy infection. The blood was collected asceptically, defibrinated and centrifuged. The parasites were found to occupy a grey layer between the white leucocytic zone and the red layer of non-infected R.B.C. They were separated and dilutions were made with physiological saline containing 0.5 per cent. phenol which was used for preservation, and for killing the parasites.

EFFECT ON PARASITE RATE

The parasite count was made against nucleated R.B.C. of Pigeons and the figures, tabulated, show the number of malaria parasite per c.mm. of blood.

Group I consisted of 2 monkeys, M2 and M15, were cases of chronic infection. In M2 an initial injection of 70 million parasites caused a gradual fall in the parasite rate from 7,000 to 150 on the 13th day with a temporary increase on the 2nd day to 9,500.

A second injection of 210 million parasites caused the disappearance of the parasites from the peripheral circulation in 8 days but after an absence for 14 days they gradually reappeared to a number of 140 when a third injection of 100 million parasites was given. This again was sufficient to remove the parasites from the peripheral circulation for at least 24 days beginning on the 9th day after the injection.

In M15 an injection of 50 million parasites resulted in a fall of parasite count from 28,600 to 2,500 on the 5th day and to 175 on the 25th day. A second injection of 100 million parasites caused a rise in parasite rate to 5,090 on the 2nd day and a fall to zero for 5 days from the 9th to the 13th day. The parasite rate, however, returned to 2,000 on the 25th day when 150 million parasites were injected. 6 days after the 3rd injection, no parasite could be found in the peripheral blood. This condition persisted for 10 days after which parasite reappeared in the peripheral blood.

Group II consisted of 2 monkeys, M20 and M21, with primary acute infection. 10 million parasites were injeted into the both just after the peripheral blood films were positive for *Plasmodium knowlesi*. Two other doses of 25 millions and 50 millions parasites were injected 3 days and 6 days after the 1st injection. The parasite rate, however, increased steadily. One of the subjects died while the other was bled. The parasite rates have been indicated by +, + +, + + +, + + + +.

EFFECT ON BLOOD COUNT

The total number of leucocytes slightly increased for 4 to 7 days after each injection and remained so for 10 to 12 days, coming down to normal very rapidly. In the differential count, the lymphocytes, with a preponderance of the medium sized ones, showed a large increase from 48% to 76% in M2 and from 55% to 75% in M15. Compared with these the monocytes showed a slight increase, the maximum number being 5% in M2 and 6% in M15. In M20 and M21, the monkeys with acute infections, parallel results were obtained. In both there was slight leucopenia during the last stages of infection, the fall being from 6,400 per c.mm. before infection to 5,200 in M20 and from 5,800 per c.mm. to 4,500 per c.mm. in M21. In cases of differential counts again, the number of lymphocytes increased from 45% to 81% mostly at the expense of neutrophils whose number fell from 51% to 15% in case of M20. In M21 the lymphocytes increased from 50% to 68% and the

neutrophils fell from 45% to 28%. The monocytic change was not marked. In M20 the number was 6% in the height of acute infection while in M21 it was 4%.

TABLE I

Showing the effect of the antigen on monkeys of Group I.

Day	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Total leucocyte per c.mm.	M.P. per c.mm.	Antigen administered.
M2.							
1	48	48	2	2	7,800	7,000	70 mill
2	52	46	2	0	8,000	9,500	
4	30	60	4	6	8,500	1,700	
5	25	70	3	2	10,700	1,070	
13	26	67	4	3	7,500	150	
14	20	76	3	1	5,000	280	210 ,,
18	35	66	3	1	8,300	132	
22	25	68	4	3	8,400	0	
27	25	71	2	2	8,400	0	
35	30	67	3	0	6,400	0	
38	41	52	1	6	6,500	55	
45	44	50	4	2	6,200	140	100 ,,
48	28	69	2	1	5,800	30	
54	32	63	4	1	8,300	0	
58	22	73	4	1	7,500	0	
64	20	73	5	2	6,800	0	
72	28	68	3	1	6,200	0	
76	43	55	1	1	6,800	25	
M15							
1	39	55	5	1	8,200	28,800	50 ,,
5	20	73	6	1	9,800	2,500	
9	22	75	3	0	5,200	2,000	
17	31	63	4	2	5,300	215	
25	34	60	4	2	3,500	175	
27	30	65	3	2	5,600	5,090	100 ,,
29	24	70	5	1	5,600	1,400	
30	16	79	3	2	7,200	400	
34	30	66	3	.1	5,200	176	
38	25	70	5	0	6,400	0	
42	30	65	2	3	6,400	200	
49	41	57	2	0	5,800	700	
52	58	40	1	1	4,800	2,000	150 ,,
55	34	60	3	3	8,300	110	
58	22	72	4	2	7,600	0	
65	31	64	3	2	5,600	0	
69	45	52	3	0	5,200	11	

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TABLE II

Showing the effect of the antigen on monkeys of Group II.

Day	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Total leucocyte per c.mm.	M.P. per c.mm.	Antigen administered.
M20.							
Infected I.V.	51	45	2	2	6,400	o	
7	42	50	6	2	7,000	+	10 mill
10	24	70	6	o	5,400	+ +	25 ,,
12	15	81	4	o	5,200	+ + +	50 ,.
13	Bled						
M21							
Infected I.V.	45	50	2	3	5,800	o	
5	40	55	2	3	6,800	+	10 ,,
9	31	65	3	1	5,800	+ + +	25 ,,
11	28	68	4	o	4,500	+ + + +	50 ,,
12	Died						

CONCLUSION

It is known that any chronically infected subject can show wide variations in parasite rate but the remarkable way in which the parasite count came down after every injection or disappeared from the peripheral circulation, lends support to the view that the effect was due to the antigens introduced. It was also evident that the increased lymphocytic count coincided with the decrease in the number of the parasites in the circulation, the antigen having stimulated the lymphocyte forming organs.

The failure of the vaccine on the parasite count in acute cases can be explained by the fact that the defence mechanism was so quickly overwhelmed by the invasion of the parasites that no further stimulus could help it in its fight.

My best thanks are due to Dr. J. C. Ray for his kind interest and advice.

OBSERVATIONS ON IN-VITRO CULTIVATION OF *PLASMODIUM
KNOWLESI* BY A MODIFIED BASS AND JOHNS METHOD

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Since the publication of the Bass and Johns method (1) about a quarter of a century ago, numerous reports based on the original technique or on its several modifications, have been published. Some have claimed very encouraging results of several generations of the parasite either in the original culture or in the subcultures, while other workers have strongly denied it. The successful cultivation of malaria parasites is of paramount importance for a study of biology of the parasites and also for the treatment and prevention of malarial attacks. It is believed that a successful method for cultivating any of the Plasmodia may need little change when applied to others of the same genus. In the present paper are recorded the results of the experiments made for determining three questions:

1. Is there any increase in the total number of the parasites?
2. Are the merozoites liberated and do they attack fresh R.B.C.?
3. What is the optimum temperature for cultivation?

MATERIALS AND METHODS

Blood was withdrawn with a sterile syringe from a rhesus monkey chronically infected with *Plasmodium knowlesi* and put inside a sterile flask with glass beads and defibrinated by shaking. 0.03 c.c. of 50 per cent glucose solution was added for every c.c. of blood. This blood, with added glucose, was distributed into sterile Kahn tubes 2 c.c. in each tube and incubated.

1. *Total number of parasites :*

Primary count was made in the following manner. Defibrinated blood with added glucose was mixed with equal quantity of a standardised suspension of nucleated red blood corpuscles of pigeon and smears were made from the mixture. After 24 hours the contents of each tube were mixed thoroughly and similar smears made with nucleated R.B.C. suspension. After 48 hours another set of films were made from a different number of tubes. The parasites were counted against one thousand nucleated R.B.C. Incubation was done at 37°C.

2. *Merozoites :*

Control films were made from the blood in the flask. Every three hours, smears were made from the top layer of the R.B.C. in the Kahn tubes, and at each time, smears were also prepared from fresh monkey blood for comparison.

3. *Optimum temperature :*

Only two temperatures were compared, 24°C and 37°C. At the end of every 24 hours, monkeys were infected with the culture material. Testing the monkeys for result was carried on for 8 weeks from the date of inoculation.

RESULT

1. *Total number :* There were slight increase in the total number upto 40 per cent to 60 per cent of the primary count, at the 24th hour but after the 48th hour a notable decrease was observed.

2. *Merozoites :* The first liberation of merozoites occurred simultaneously in the blood of the monkey and in the blood in the Kahn tubes. Films made at that period clearly showed free merozoites. Some of the parasites were found in a very early stage of ring form, some were found in the accole position and in some the cytoplasmic vacuole was not yet evident. There was no phagocytosis by any of the leucocytes. The pictures tallied exactly with those of the smears made from fresh monkey blood. In the next smears made 3 hours

later, excepting the very few free merozoites, the youngest forms were all intracellular tiny rings. No phagocytic condition was observed. In the subsequent smears free merozoites could still be found, although rarely. After a lapse of about 18 to 22 hours from the time of the first completion of schizogony, merozoites could again be seen developing into ring form inside the red blood corpuscles. But this time they form a very small percentage of the total number of parasites.

3. *Other forms of parasite and red blood corpuscles :* Degeneration started early, sometimes noticeable even at the 12th hour. The forms that suffered most were the schizonts. Irregular staining of both the chromatin and the cytoplasm whose continuity was also disturbed, occurred. On the whole, the appearance were like those of the parasites after the administration of quinine. Degenerative changes were also noticed in the trophozoites. By the 24th hour the number of such degenerated forms varied from 10 to 15 percent and by the 48th hour this number reached upto about 90 percent. Hæmolysis of red blood corpuscles began even at the 24th hour and a few extracellular forms, with degenerative changes, noted at that period, was due to such a change in the medium. As more time elapsed there was more hæmolysis and consecutive appearance of such forms in larger number.

4. *Optimum temperature :* Monkeys could be successfully infected with culture material kept at 37°C for not more than 2 days whereas when incubated at 24°C it was possible to infect them with 4 days old culture.

DISCUSSION

The comparatively slight increase in the total number of the parasites at the 24th hour was the result of the accompanying degenerative process and also perhaps of the artificial conditions under which fewer parasites could sporulate. Phagocytosis played little or no part in it. At the 48th hour about 90 percent of the parasites were degenerated and the marked decrease in their total number was due to such rapid degeneration and lysis.

The three hourly preparations in many cases revealed free merozoites in the film and also the presence of several merozoites which had just entered or were about to enter the red blood corpuscles. The presence of a few merozoites in the subsequent films was due to their failure to enter any R.B.C. The antibodies in the serum may be responsible for this. Phagocytosis by the leucocytes were rather rare although no degeneration was noticed in them for about 18 hours. The second shower of merozoites occurred from 18 to 22 hours after the first and under the given conditions very few of the parasites could complete the Schizogony cycle.

In each case successful infection of the monkeys proved that the para-. sites were still alive. It was found that under identical conditions with only the temperature varying, the parasites could live much longer at the cooler temperature of 24°C than at 37°C.

CONCLUSION

1. When Plasmodium knowlesi is cultivated according to the method described above, liberation of merozoites occur with the same periodicity in the culture tubes, as in the susceptible host.

2. Liberated merozoites can attack fresh red blood corpuscles and at least a few can complete the schizogony cycle in R.B.C.

3. Degeneration of the parasites sets in early, noticeable even at the 12th hour.

4. Parasites remain viable for a longer period at 24°C than at 37°C.

Our best thanks are due to Dr. J. C. Ray for his kind interest and advice.

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ROLE OF PHOSPHORUS, FLAVIN AND HORMONES
IN THE UTILISATION OF PROTEINS

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It has only recently been recognised that various factors, present in the diet may be concerned in the utilisation of dietary proteins. Basu and Gupta (1) have carried out systematic work on the rôle of the different vitamins on the biological value of proteins.

Some amount of work has also been performed on the rôle of minerals. The claim made by Ranganatham and Rao (2) that the addition of calcium to the calcium deficient diet considerably increases the ·digestibility and biological value of the proteins has been refuted by Basu and Gupta in this laboratory (*loc. cit.*).

Working with sheep Woodman and Evans (3) found that the digestibility of the ration was unchanged by either calcium or phosphorus deficiency. Similar results were obtained by Kleiber, Goss and Gulbert (4) with steers and a diminished utilisation (20 per cent. less) of food energy was also observed by them. Recently Morris and Ray (5) have studied the effect of phosphorus deficiency on nitrogen metabolism of sheep. They conclude: "It is doubtful if there is any significant difference in the apparently digestibility of the protein ingested. There is, however, no doubt that a deficiency of phosphorus in the diet causes a significant lowering of the true digestibility of the proteins." They also obtained an appreciably lower figure for the biological value of proteins of blood meal with the phosphorus deficient diet.

It was also suggested long ago that conditions modifying the glandular activities of the body (an excessive or diminished secretion) might change the measured biological value of proteins. But no systematic study has yet been made to clear up this point. The effect of the anterior pituitary hormone on general nitrogen metabolism has been studied by Gæbler and Price (6) and by Shaffer and Lee (7). Some work has also been done with thyroxin (8) and insulin (9). All these investigations show that the excretion of nitrogen is markedly increased during the administration of the hormone preparations.

The aim of the present investigation was to find out the effects of the absence of flavin, and of phosphate in the diet on the utilisation of protein

by the balance sheet method and also to observe the effects produced by the hormone preparations on protein metabolism.

EXPERIMENTAL

The experiments were carried out with adult rats and the experimental diets always contained 10% protein.

The technique previously employed by Basu and his collaborators (10, 11, 12, 13) was also followed in this case. The nitrogen-free diet consisted of 12 parts ghee (butter-fat), 9 parts chopped sugar, 5 parts salt mixture, 2 parts cod liver oil, 1 part calcium carbonate and 71 parts starch.

ROLE OF PHOSPHORUS

In determining the rôle of phosphorus in protein utilisation, the following procedure was adopted. First experiments were made with a complete diet (A), containing all the essential ingredients in adequate amounts. Next determinations were carried out with a diet (B) otherwise complete, except that its phosphate content in the salt mixture was only one-third. The third set of experiments was performed with a diet (C) containing no phosphate in the salt mixture. The proteins chosen for this investigation were vitamin-free casein and egg-albumin. It is to be noted that while casein itself contained phosphorus, egg-albumin was free from this element. The digestibility and biological value of proteins on complete diet, phosphorus deficient diet and phosphorus-free diet are given in Tables II and III.

The endogenous urinary and fæcal nitrogen for each rat was determined by feeding the rats on nitrogen-free diet before and after the series of experiments and the mean values are given in Table I.

TABLE I

Experiment with nitrogen-free ration.
(Figures for intake and excretion represent daily averages).

Rat No.	Average weight. (g.)	Food intake. (g.)	Urinary nitrogen. (mg.)	Fæcal nitrogen.	
				Total (mg.)	Per g. of food intake. (mg.)
701	249	10.78	57.6	20.98	1.99
702	282	11.22	59	20.78	1.85
703	260	10.3	57.5	25.75	2.3
704	146	8.95	46	23.37	2.7
705	147	8.3	38	16.6	2
706	206	9.92	49.8	17.95	1.82
707	203	11.55	70	23.1	2
708	224	12.1	66	21.78	1.8
709	248	10.73	74	21.4	1.95
710	220	11.5	62.3	26.45	2.3
711	170	10.8	69.2	21.6	2
712	179	9.57	60.7	20.1	2.1

TABLE II

Effect of phosphorus deficiency on biological value and digestibility of casein.

(Figures for intake and excretion represent daily averages).

A. Complete diet.

Rat No.	Average wt. (g.)	Intake		Faecal nitrogen		Food N absorbed. (mg.)	Urinary nitrogen		Food N utilised. (mg.)	Biological value.	Mean B.V.	Digesti-bility.
		Food. (g.)	Nitrogen. (mg.)	Endo. (mg.)	Exo. (mg.)		Endo. (mg.)	Exo. (mg.)				
701	250	6.4	102.5	12.7	9.98	92.52	57.6	29.4	63.12	68.2	68.9	90
702	284	10.48	167.7	19.39	11.5	156.2	59	49	107.2	68.6		93.2
703	262	9.7	153.9	24.25	9.7	144.2	57.5	45.2	99	68.6		93.7
704	150	7.56	121	20.41	8.7	112.3	46	37.3	75	66.7		92.8
705	149	7.7	124.8	15.4	7.5	117.3	38	34.3	83	70.8		94
706	208	3.85	61.5	7	2.87	58.63	49.8	17.2	41.43	70.7		95.3

B. Phosphorus-deficient diet.

Rat No.	Average wt. (g.)	Intake		Faecal nitrogen		Food N absorbed. (mg.)	Urinary nitrogen		Food N utilised. (mg.)	Biological value.	Mean B.V.	Digesti-bility.
		Food. (g.)	Nitrogen. (mg.)	Endo. (mg.)	Exo. (mg.)		Endo. (mg.)	Exo. (mg.)				
707	205	13.55	217.5	27.1	17.2	200.3	70	109.5	90.8	45.3	46.8	92.1
708	226	12.3	197	22.14	15.3	181.7	66	92.3	89.4	49.2		92.2
709	250	9	144	17.55	14	130	74	70.2	59.8	46		90.3

C. Diet containing phosphorus-free salts.

Rat No.	Average wt. (g.)	Intake		Faecal nitrogen		Food N absorbed. (mg.)	Urinary nitrogen		Food N utilised. (mg.)	Biological value.	Mean B.V.	Digesti-bility.
		Food. (g.)	Nitrogen. (mg.)	Endo. (mg.)	Exo. (mg.)		Endo. (mg.)	Exo. (mg.)				
701	282	11.92	191	23.8	16.2	174.8	57.6	113.4	61.4	35.1	36.1	92
702	307	10.02	160.3	19.04	23.96	136.34	59	81	55.34	40.6		85.2
703	282	10.7	171.2	26.75	21.3	149.9	57.5	94.5	55.4	37		87.6
704	159	8.4	134	22.68	12.2	121.8	46	71	50.8	41.7		90.9
705	158	8	128	16	13.6	114.4	38	67	47.4	41.4		89.4
706	212	7.2	115	13	10	105	49.8	62.2	42.8	40.8		91.3

TABLE III

Effect of phosphorus deficiency on biological value and digestibility of egg-albumin.

Rat No.	Biological value.	Mean B.V.	Digestibility.
A. *Complete diet.*			
711	74.4	73.2	96.2
712	72.1		96.8
B. *Phosphorus-deficient diet.*			
710	60	60.2	96.7
711	58.1		96.9
712	62.6		96.2
C. *Phosphorus-free diet.*			
707	48.6	51.7	95.8
708	51.9		96.1
709	54		97.1
710	52.2		95.4

EFFECT OF ABSENCE OF LACTOFLAVIN

In all determinations of biological value, vitamin-B complex is generally added to the ration in the form of marmite (yeast extract) solution. In order to show the effect of flavin omission on biological value, three series of experiments were performed. First series of determinations were carried out with the usual diet (casein, at 10% level) using marmite extract as source of vitamin-B complex which, however, was not used during the last two series of experiments. In the second series of experiments, vitamin B_1 only was added (10 mg. of international vitamin B_1 preparation per rat per day). In the third series of experiments lactoflavin (10 μ per rat per day) was given in addition to 10 mg. of vitamin B_1. The results are shown in Table IV.

The endogenous urinary and faecal nitrogen for each rat is given in Table I.

TABLE IV

Effect of flavin on biological value and digestibility of casein (10% level).

A. Complete diet.

Rat No.	Biological value.	Mean B.V.	Digestibility.
701	68.2	68.4	90
702	68.6		93.2

TABLE IV (*Contd.*)

B. Diet containing 10 mg. of vitamin B_1 (international) powder, supplied to each rat per day, other factors of vitamin B-complex being absent.

Rat No.	Biological value.	Mean B.V.	Digestibility.
707	47.3	44.9	89.6
708	45.2		87.6
709	42.4		92.4
710	45.4		92.1
711	41.6		89
712	46.4		94.8

C. Diet containing 10 mg. of vitamin B_1 powder and 10 μ of lacto-flavin given to each rat per day.

Rat No.	Biological value.	Mean B.V.	Digestibility.
707	56.6	58.2	96.1
708	56.5		95.2
709	54.7		96.1
710	58.4		94.8
711	62.8		94.7
712	60		91.5

EFFECT OF ADMINISTRATION OF HORMONES

The dietary protein used in these investigations was casein at 10% level.

Adrenaline.—Adrenaline chloride solution (0.1 cc. of 1:1000 in physiological saline) was given to each rat each day. Results are indicated in Table V.

Thyroxin.—A standard preparation of thyroid gland was administered to the diet of experimental rats, the dosage being 0.5 g. per rat per day. Results are shown in Table VI.

Anterior pituitary.—A standard preparation was administered to the diet of the experimental rats, the dosage being 0.5 g. per rat per day. Results are indicated in Table VII.

TABLE V
Effect of adrenaline on biological value and digestibility of casein.

Rat No.	Biological value.	Mean B.V.	Digestibility.

A. *Complete diet without adrenaline.*

Rat No.	Biological value.	Mean B.V.	Digestibility.
703	68.6	69.7	93.7
705	70.8		94

B. *Complete diet with adrenaline.*

Rat No.	Biological value.	Mean B.V.	Digestibility.
713	38.1	34.9	93.7
714	33		92.4
715	32.8		93.3
716	35.8		92.9
717	35		93
718	34.4		94.6

TABLE VI
Effect of thyroxin on the utilisation of casein.

Rat No.	Biological value.	Mean B.V.	Digestibility.

A. *Complete diet without thyroxin.*

Rat No.	Biological value.	Mean B.V.	Digestibility.
702	68.6	69.7	93.2
705	70.8		94

B. *Complete diet with thyroxin.*

Rat No.	Biological value.	Mean B.V.	Digestibility.
719	37.2	39.3	87.6
720	39.9		88.4
721	38.4		89.8
722	41.8		86.6

TABLE VII
Effect of anterior pituitary on biological value and digestibility of casein.

Rat No.	Biological value.	Mean B.V.	Digestibility.

A. *Complete diet without anterior pituitary.*

Rat No.	Biological value.	Mean B.V.	Digestibility.
702	68.6	68.6	93.2
703	68.6		93.7

B. *Complete diet with anterior pituitary.*

Rat No.	Biological value.	Mean B.V.	Digestibility.
723	47.1	47.5	91.2
724	50.6		91.6
717	46.3		89.0
718	46.0		88

DISCUSSION

Effect of Phosphorus.—It will be observed from Tables II and III that partial or complete removal of phosphorus from the diet has practically no effect on the digestibility of proteins. The observation of Morris and Ray (*loc. cit.*) made with the sheep that a deficiency of phosphorus in the diet causes a significant lowering of the true digestibility of the proteins could not thus be confirmed in the case of rats. It appears, therefore, that phosphorylation is not a preliminary step in the absorption of amino-acids as is the case with carbohydrates and fats. But when it comes to the question of utilisation of absorbed amino-acids phosphorus appears to play a very important rôle. When the amount of phosphate in the diet is low, the biological value of both casein and egg-albumin falls appreciably and when inorganic phosphorus is completely withdrawn it falls still further. The decrease in biological value of the proteins is very probably due to the greater deamination of the absorbed amino-acids. The hypothesis of Morris and Ray (*loc. cit.*) that Ca/P ratio plays a rôle here is very unlikely in view of the observation of Basu and Gupta (1) that even complete removal of calcium from the diet had no effect on the biological value of proteins.

Effect of Lactoflavin.—Table IV makes it clear that withdrawal of flavin from the diet affects both the digestibility and biological value of proteins. The digestibility is not lowered to the same extent as the biological value.

Effect of Administration of Hormones.—Tables V, VI and VII show that the administration of adrenaline chloride or of thyroid gland or of anterior pituitary preparation, while not appreciably affecting the digestibility of casein, very much lowers its biological value. The effect is more marked with thyroid gland preparation and adrenaline than with the anterior pituitary preparation. Thyroid gland secretion greatly increases metabolism including that of the amino-acids. This results in a greater deamination of the amino-acids and hence in a greater elimination of nitrogen. The biological value is thus lowered. Adrenaline also appears to stimulate amino-acid metabolism and thus to lower the biological value of proteins. The effect of anterior pituitary is very probably an indirect one ; it acts by stimulating other glands like the thyroid and adrenals to greater activity.

SUMMARY

Partial or complete removal of phosphorus from the diet has no effect on the digestibility of proteins but lowers the biological value. Phosphorylation is thus not a preliminary step in the absorption of amino-acids but is of importance in the utilisation of absorbed amino-acids for protein formation.

Withdrawal of flavin lowers both the digestibility and biological value. Administration of adrenaline chloride, thyroid gland or anterior pituitary preparation, while not affecting the digestibility, very much lowers the biological value.

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BIOLOGICAL VALUE OF THE PROTEINS OF PAPAYA (*CARICA PAPAYA*) AND LADY'S FINGER (*HIBISCUS ESCULENTUS*)

K. P. BASU AND H. P. NATH

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The present investigation is concerned with the determination of the nutritive value of the proteins obtained from papaya (*Carica papaya,* Linn) and lady's finger (*Hibiscus esculentus*) by the nitrogen balance method using adult rats as experimental animals.

EXPERIMENTAL

Firstly the moisture contents of papaya and of lady's finger were determined, and then the protein content of the dried mass was found out. Papaya contained 92% moisture and 1% protein and the dried material contained 12.9% protein. The moisture and protein content of lady's finger were 90% and 2.2% respectively; the dry material contained 21.7% protein. The technique employed for determination of biological value was the same as used in previous investigation (1). The experimental diet contained 9% proteins in the case of *Carica papaya* and 10% in the case of *Hibiscus esculentus* diet. The metabolic data are presented in Tables I and II.

DISCUSSION AND SUMMARY

It will be observed from Tables I and II that the proteins of both *Carica papaya* and *Hibiscus esculentus* are of a very poor quality and they are even inferior to most of the pulse proteins investigated by Basu and his co-workers. The biological values of the proteins of the better quality pulses lie between 50 and 60 and their digestibility also is slightly higher than that of proteins from these two sources. As sources of proteins therefore, neither *Carica papaya* nor *Hibiscus esculentus* is of any great importance.

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TABLE I

Digestibility and biological value of Carica papaya *at 9% protein level.*
(Figures for intake and excretion represent daily averages).

Rat No.	Average wt. (g.)	Intake Food (g.)	Intake Nitrogen (mg.)	Faecal nitrogen (mg.) per g. of food intake	Faecal nitrogen Endo. (mg.)	Faecal nitrogen Exo. (mg.)	Food N absorbed (mg.)	Urinary nitogen Endo. (mg.)	Urinary nitogen Exo. (mg.)	Food N utilised (mg.)	Biological value	Mean B.V.	Digesti-bility
719	157	13.9	200.16	1.9	26.41	22.15	178	41.2	97.9	80.1	45	46	88.9
720	171	11.37	163.8	1.7	19.33	23.7	140.1	38.6	76.8	63.3	45		85.5
721	193	13.4	193	2.05	27.47	26.5	166.5	50	87.1	79.4	48		86.3
722	148	9.95	143.2	1.8	17.91	18.6	124.6	27.5	67.3	57.3	46		87

TABLE II

Digestibility and biological value of Hibiscus esculentus *at 10% protein level.*
(Figures for intake and excretion represent daily averages).

Rat No.	Average wt. (g.)	Intake Food (g.)	Intake Nitrogen (mg.)	Faecal nitrogen (mg.) per g. of food intake	Faecal nitrogen Endo. (mg.)	Faecal nitrogen Exo. (mg.)	Food N absorbed (mg.)	Urinary nitogen Endo. (mg.)	Urinary nitogen Exo. (mg.)	Food N utilised (mg.)	Biological value	Mean B.V.	Digesti-bility
717	207.2	13.54	216	2	27.08	32.4	181.1	45	104.8	79.3	43	42	85.2
718	192	11.5	184	2.2	25.3	27	157	45	90.1	66.9	43		85.3
723	187	12.5	200	2.4	30	33.5	166	45	93.2	72.8	44		83.0
724	190	10.86	173.8	2.3	24.98	27.3	146.5	56	87.9	58.6	40		84.3

COLOUR REACTIONS OF STEROIDS IN RELATION TO THEIR STRUCTURES

M. C. NATH AND M. K. CHAKRABORTY

From the Physiological Section, Chemical Laboratories, Dacca University.

That unsaturation plays an important rôle in the development of colour reactions of sterols, has been established by many workers (1). It was noticed by Rosenheim (2) that his reagent produces an immediate red solution with ergosterol in chloroform (band at 500 mμ) which gradually changes to blue (bands at 570-580 and 650-680 mμ) on keeping. He also observed that other natural sterols when purified do not show any change of colour with his reagent at ordinary temperature, but on warming the solution turns red (band at 500 mμ), no blue phase being developed. This led him to suppose that change of colour from red to blue is specific for ergosterol only. The production of red colour was also supposed to be related to the ethenoid

linkage at C_1-C_{13} or C_1-C_2 of the old sterol skeleton. Similar red colouration was also observed (3) with ψ-cholestene, *allo*cholesterol and with cholesterilene (4).

Kohlenberg's reaction, which was also supposed to be specific for ergosterol and its derivatives, has been found by Heilbron and Spring (5) to be positive with cholesterilene, ψ-cholestene, *allo*cholesterol and β-cholesterol, the colour varying from the red to reddish brown.

Rosenheim and Callow's reagent (6), though inactive towards cholesterol, cholesteryl chloride, cholestenone, oxycholestenone, dihydrocholesterol, coprosterol, has been found to give red colours with cholesterilene, ψ-cholestene, *allo*cholesterol and β-cholesterol (selective absorption at 500 mμ) in each case. But ergosterol and its esters behave in a different manner ; the colour developed is blue and there is no selective absorption.

Tortelli-Jaffe's reaction (7) which is positive with ergosterol, α- and β-dihydroergosterol, α-ergosterol, and β-*iso*ergosterol and dehydroergosterol, is negative towards β-ergosterol, cholesterol and all its derivatives. This reaction was taken by Heilbron and Spring (5) to be specific for sterol derivatives containing an inert linkage (as applied to hydrogenation). They supposed that this ethenoid linking might be in the position C_{10}-C_{19} of the old sterol formula.

It is thus seen that, with the exception of a few, many steroids develop red colouration in solution with different reagents. It seems that this peculiar phenomenon of colour production may not be without any significance and may be governed by some specific rule. But as no systematic attempt has hitherto been made to explain the relations between the colour reactions and the accepted structure of sterols, it was thought that an investigation in this line would be of interest, particularly in the study of the constitution of steroids.

The following table records the colours developed by Rosenheim's, Kohlenberg's and Rosenheim and Callow's reagents on some sterol derivatives.
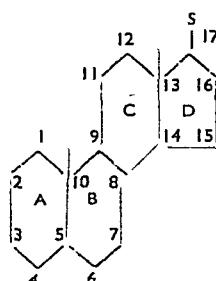
(Formula I)
Sterol skeleton.

TABLE I

Substance.	Formula (I).	Change of colours by		
		Rosenheim's reagent.	Kohlenberg's reagent.	Rosenheim and Callow's reagent.
1. *allo*Cholesterol	OH at C_3 Double bond at 4:5 $S = C_8H_{17}$	Yellow → orange	Brown red	Red
2. Cholesterilene	Double bonds at 3:4 and 5:6 $S = C_8H_{17}$	Carmine red	Carmine red	Red
3. ψ-Cholestene	Double bond at 3:4 $S = C_8H_{17}$	,, ,,	Brown red	Carmine red
4. Cholesterol	OH at C_3 Double bond at 5:6 $S = C_8H_{17}$	Negative	Negative	Negative
5. 7-Dehydrocholesterol	Double bonds at 5:6 and 7:8 $S = C_8H_{17}$	Red → blue	Pink → blue	—
6. α-Dehydroergosterol (Ergostadiene 8:14, 22:23 −ol.3)	OH at C_3 Double bond at 8:14 $S = C_9H_{17}$	Negative	Negative	Negative
7. Ergosterol	OH at C_3 Double bonds at 5:6 and 7:8 $S = C_9H_{17}$	Pink → blue	Red → blue → green	Pink → blue
8. β-Dihydroergosterol	OH at C_3 Double bond at 14:15 $S = C_9H_{17}$	Negative	Negative	Negative
9. α-Ergostenol	OH at C_3 Double bond at 8:4 $S = C_9H_{19}$	,,	,,	,,
10. β-Ergostenol	OH at C_3 Double bond at 14:15 $S = C_9H_{19}$	,,	,,	,,
11. *iso*Ergostenol	OH at C_3 Double bonds at 4:5 and 6:7 $S = C_9H_{17}$	Pink	—	Red → purple → blue → olive green
12. Dehydroergosterol	OH at C_3 Double bonds at 5:6, 7:8 and 9:11 or 14:15 $S = C_9H_{17}$	Pink → green	Blue	—

In accordance with the present conception of the sterol structure the statement of Rosenheim (*loc. cit.*) assumes the following form:

The production of an immediate carmine red or red colouration is dependent upon the presence of 3 : 4 or 4 : 5 ethenoid linkage.

The observation of Rosenheim (*loc. cit.*) that cholesterol on being warmed with his reagent develops carmine red colour gives additional support to this view. He has also stated that cholesterol under this condition is changed to *allo*cholesterol having the Δ 4:5 linkage, which is consequently responsible for development of the carmine red colour.

Windaus observed that the double bond in cholesterol can be shifted from the $\beta\gamma$ to the $\alpha\beta$ position with respect to the OH-group by means of hydrochloric acid. We have found that the substance formed by passing anhydrous hydrochloric acid gas through a chloroform solution of cholesterol for about two hours at ordinary temperature, gives positive reaction with Rosenheim reagent. This also shows that the Δ 4:5 linkage is in some way related to the development of carmine red colouration.

Further confirmation of this may be obtained from the application of our new colour reaction for steroids (8) to cholic and desoxycholic acid (Table II).

TABLE II

Substance.	Reagent used.	Colour of the rings.
Cholic acid (in glacial acetic acid)	2% Acetic acid solution of mercuric acetate (one drop) and a few drops of conc. sulphuric acid.	No ring immediately; a fine blue ring appears in about 20 seconds which gradually turns into brilliant violet in 2-3 minutes and becomes carmine on long standing.
Desoxycholic acid (in glacial acetic acid)	,, ,,	A brilliant violet ring after about 20 seconds; no blue ring or layer is fomed.

DEVELOPMENT OF BLUE COLOUR

It should be noted that a dehydrating agent (such as concentrated sulphuric acid) is required in our reaction which acts by eliminating a molecule of water and an additional double bond is thus created. Rosenheim and Callow found (*loc. cit.*) that cholatrienic acid prepared by distillation of cholic acid (9) gives gentian blue colouration with their reagent while choladienic acid is indifferent. These observations may lead to three views:

(1) In course of our colour reaction cholic and desoxycholic acids are possibly changed to cholatrienic (II) and choladienic acids (III) respectively.

Cholatrienic acid (II).
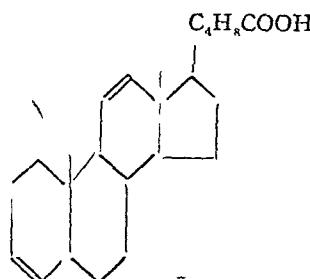
Choladienic acid (III).

(2) The double bond at C_{11}-C_{12} has previously been shown (10) to be non-reactive to chemical reagents. Hence it is likely that the $\Delta 7 : 8$ linkage is closely related to the development of blue colouration in all the reactions (compare substances No. 5, 7 and 12 in Table I).

It is really striking that in all those cases where there is a 7 : 8 linkage the development of blue phase follows the pink or red.

Substances No. 6, 8, 9 and 10 (Table I), where there is a linkage either in the positions, 3 : 4, 4 : 5 or 7 : 8, no red or blue colour is to be expected according to this hypothesis ; and this was found to be the case

The observation of Rosenheim (*loc. cit.*) that cholesterol on being warmed with his reagent develops carmine red colour gives additional support to this view. He has also stated that cholesterol under this condition is changed to *allo*cholesterol having the $\triangle$ 4:5 linkage, which is consequently responsible for development of the carmine red colour.

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C_4H_8COOH C_4H_8COOH

Cholatrienic acid (II).   Choladienic acid (III).

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It is really striking that in all those cases where there is $\Delta 7:8$ linkage, the development of blue phase follows the pink or red.

Substances No. 6, 8, 9 and 10 (Table I), where there is no double bond either in the positions, 3 : 4, 4 : 5 or 7 : 8, no red or blue phase should be expected according to this hypothesis ; and this has actually been found to be the case.

CHANGE OF COLOUR FROM RED TO BLUE

It has been shown by Windaus and Uiberig (11) that cholesterol can be transformed into cholestanol by hydrogenation in presence of Willstätter's platinum catalyst and a trace of hydrochloric acid which causes shifting of the double bond at C_5-C_6 to C_4-C_5. From the location of the double bonds in ergostadiene ($\Delta 8:14$, $\Delta 22:23$) and its β-form ($\Delta 14:15, \Delta 22:23$) (12) it is seen that in course of reduction, the double bond at C_7-C_8 is shifted to C_8-C_{14} or C_{14}-C_{15} ; while the double bond at C_5-C_6 (which is probably first shifted to 4 : 5 position) gets saturated.

It is thus clear that $\Delta 4:5$ is the most reactive of all the double bond towards hydrogenation and may also be so towards colour developing agents.

It has already been pointed out that $\Delta 4:5$ is associated with the development of red or carmine red colouration. Where there is a second

double bond at C_7-C_8 the blue colour is developed gradually and the intensity of red is decreased probably due to further shifting of the bond a C_4-C_5 to some other inert position and thus the gentian blue colour soon makes its prominence.

FINAL GREEN TINGE IN STEROL REACTION

In case of Rosenheim's reaction with dehydroergosterol (Windaus añd Linsert, 13) the colour rapidly changes to green, no blue phase being observed at all (Table I, No. 12). This would signify that the additional double bond ($\Delta\,9:11$ or $\Delta\,14:15$) created during dehydrogenation might cause the appearance of green colour. Heilbron and Spring (14) have shown that a system of conjugated double bond is necessary for giving positive colour reaction with Rosenheim's reagent. In dehydroergosterol, the additional double bond $\Delta\,9:11$ or $\Delta\,14:15$ is conjugated with $\Delta\,7:8$.

CONJUGATION OF DOUBLE BONDS

It will be interesting to discuss whether this system of conjugation is always necessary for Rosenheim's as well as our colour reaction.

Desoxycholic acid which is probably changed in choladienic acid with our reagent gives brilliant violet ring within about 20 seconds on adding the acid. Here there is little possibility of the formation of conjugated double bonds. Cholatrienic acid, though it possesses no such system of double bonds, is positive towards Rosenheim and Callow's reagent (*loc. cit.*) giving a fine gentian blue colour. Of course, one may assume here that the double bonds may rearrange themselves so as to form a conjugated system. More light can be thrown into this by our reagent. We find (Table II) that no ring is formed immediately but a fine blue ring makes its appearance after about 20 seconds which gradually changes into brilliant violet in 2-3 minutes. From this it can be assumed that by our reaction cholic acid is first changed into cholatrienic acid when (due to the presence of the $\Delta\,7:8$ linkage) there is formation of blue colour at once, but $\Delta\,3:4$ has still to be shifted to $\Delta\,4:5$ to give rise to red, which combining with blue forms the brilliant violet colouration. This accounts for the later development of violet colour nicely. The late development of violet colour may also be accounted for by assuming the formation of a conjugated system of double bonds. Cholatrienic acid first formed is possibly changed to *isocholatrienic acid* (IV) through the tautomerisation of $\Delta\,3:4$ to $\Delta\,4:5$ and $\Delta\,7:8$ to $\Delta\,6:7$, when the violet changes to carmine red. There is some difference in behaviour

regarding tautomerisation in case of bile acid, for possibly there is necessity of formation of conjugation of double bonds in the first stage of reaction.
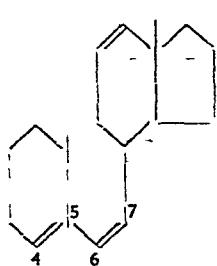
*Iso*cholatrienic acid (IV).
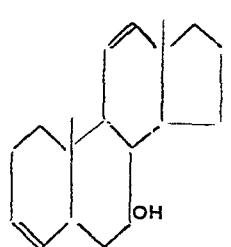
7-hydroxycholadienic acid (V).

With a view to explain the formation of violet colour in case of desoxycholic acid, one has to assume the formation of 7-hydroxycholadienic acid (V) first through the action of dehydrating agent sulphuric acid and the oxidising action of mercuric acetate.*

This product is probably further changed to substance (IV) after dehydration and shifting of Δ 3 :4 to Δ 4 :5 and of Δ 7 :8 to Δ 6 :7. Here delay in the formation of 7 :8 double bond causes no formation of blue at the beginning and thus explains well the formation of violet colour at the very outset. That the double bond Δ 11 :12 is inert has previously been recorded.

How Aldehyde Exerts its Bathychromic Effect

That the development of colour in sterol-reaction is not entirely dependent on unsaturation has also been shown by many workers. It was observed by Whitby (16) that formaldehyde has a bathychromic effect in developing the colour in a sterol solution with sulphuric acid, thus showing the possibility of formation of co-ordinate complexes. Salkowski found an orange red colouration in the chloroform layer of cholesterol on addition of sulphuric acid. But addition of a few drops of formaldehyde prior to the addition of sulphuric acid (as adopted by Whitby) developed a cherry red colouration in the chloroform layer. If one drop of acetic anhydride is added to this. layer the colour soon changes to blue and finally to green.

Since formaldehyde on warming with a drop of sulphuric acid is converted into paraformaldehyde, it was thought that this paraformaldehyde might be responsible for exerting its halochromic effect. The following table

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*This seems to be a probable change ; for very recently Wintersteiner *et al* (15) have been able to prepare 7-hydroxycholesterol only by aerating the colloidal solution of cholesterol.

3

shows the colour formation with aldehydes and sulphuric acid as applied to sterol solution under various conditions.

TABLE III

Solution of sterol used.	Reagent added to the solution	Changes of colour
1. Chloroform solution of cholesterol sulphuric acid.	(a) Formaldehyde (in excess) to the chloroform layer.	Colour disappears.
	(b) Some more sulphuric acid to (a).	Cherry red (with green fluorescence).
	(c) Solid paraformaldehyde to the orange coloured layer.	Cherry red.
2. Chloroform solution of cholesterol.	(a) Formaldehyde + sulphuric acid.	Chloroform layer, cherry red.
	(b) Acetic acid (one drop) to the cherry red layer of the reaction product of (a).	Blue (instantaneous).
	(c) Acetic anhydride in place of acetic acid in (b).	Blue (instantaneous) changing to green in half an hour.
	(i) Solid paraformaldehyde.	No colour.
	(ii) Sulphuric acid (one drop) to the product of (a).	Green changing to blue.
	(iii) Sulphuric acid solution of paraformaldehyde.	Green changing to blue.

It can be seen from this table that though formaldehyde causes disappearance of the orange red colour of the chloroform layer paraformaldehyde deepens it to cherry red, thus indicating possibility of its formation and taking part in the production of co-ordination complexes in the course of reaction.

ABSORPTION BANDS IN RELATION TO THE LOCATIONS OF DOUBLE BONDS

Rosenheim has observed a band at 500 mμ with the chloroform solution of ergosterol and his reagent (trichloroacetic acid), while developing the red phase. It is interesting to note that as the colour changed to blue, the band also shifted to 570-580 mμ and a second band appeared at 650-680 mμ. Rosenheim and Callow also observed (*loc. cit.*) band at 500 mμ in case of cholesterilene, *allo*cholesterol etc. when only red and no blue colour was developed by their reagent. As we have shown previously that developments of colours are related to the location of double bonds it can be inferred that absorption bands in some particular regions are also dependent upon the positions of double bonds in the steroids.

Table IV shows some result as obtained by us as well as by others.

TABLE IV

Substance	Reagent used	Colour	Absorption band	Data collected by
1. Cholesterol	Rosenheim's reagent and heat.	Carmine red.	500 mμ 480-509 mμ	Rosenheim. Authors.
2. Cholesterol (after passing HCl gas through chloroform solution).	Rosenheim & Callow's reagent.	Carmine blue (on keeping).	485-510 mμ 560-580 mμ	Authors. ,,
3. Cholesterol (after treatment with 8% H_2SO_4 at 50° for 6 hours).	Rosenheim & Callow's reagent.	Carmine blue (on keeping).	485-505 mμ 560-570 mμ	Authors. ,,
4. (a) Ergosterol.	Rosenheim's reagent.	Carmine blue.	500 mμ 570-580 mμ & 650-680 mμ	Authors. Rosenheim. ,,
(b) Isoergosterol	Rosenheim & Callow's reagent.	Carmine (not prominent) Blue.	500 mμ	Rosenheim & Callow.
5. Œstrogenic substance from pregnancy urine.	(i) Rosenheim's reagent.	Carmine.	475-500 mμ	Authors.
	(ii) Acetic acid and sulphuric acid.	Blue.	550-508 mμ	,,
	(iii) Acetic acid, mercuric acetate and sulphuric acid.	Carmine.	480-507 mμ	,,

From this table it can be easily inferred that absorption band at 500 m μ or in the neighbourhood is indicative of the ethenoid linkage at C_4-C_5 and that at 560-580 mμ points to the presence or development of a second double bond at C_7-C_8. Experiments No. 2 and 3 again give some interesting clue to the formation of an additional unsaturated bond at C_7-C_8 through the influence of Rosenheim and Callow's reagent (mercuric acetate and nitric acid). Creation of such a double bond in ergosterol by mercuric acetate has been reported by Achtermann (17). Thus it is possible even to predict the presence of double bonds in new sterol derivatives, only by taking recourse to colour reactions alone.

SUMMARY

(1) An attempt has been made to show the close relationship between the location of double bonds in the steroids and the colours developed by some particular reagents.

(2) It has been suggested that $\triangle$ 4 :5 linkage (actual or potential) is responsible for the development of red or carmine red colour and $\triangle$ 7 :8

linkage for blue colour in Rosenheim's reaction, Kohlenberg's· reaction and Rosenheim and Callow's reaction.   ·

(3) An attempt has also been made to find out a relation between the structure of various sterols and the corresponding absorption bands observed during the development of colour by different reagents.

Our best thanks are due to Prof. J. K. Chowdhury for his kind interest in this work.

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A NEW SENSITIVE COLOUR REACTION FOR STEROIDS

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Although various colour reactions for steroids have been developed from time to time, none of them seem to be specific for all steroids. The present author has been able to discover a new sensitive colour reaction for steroids in general. The reaction is carried out as follows:

To a solution of the steroid in glacial acetic acid (1 cc,) one drop of 1% mercuric acetate (in glacial acetic acid) is added and shaken well. To the

mixture concentrated sulphuric acid is poured down the side of the test tube very carefully. At the junction of the two layers, a brown, red or violet ring is produced, and above this a blue or green one. The colour of the upper ring may gradually extend to the acetic acid layer with a suitable concentration of the steroid. The results obtained with some steroids are given in Table I.

TABLE I

Substance in glacial acetic acid solution.	Coloured rings with sulphuric acid only.	Colour reaction with mercuric acetate + sulphuric acid.	
		Lower ring.	Upper ring.
1. Cholesterol.	Violet (faint).	Red → violet.	Blue, gradually extending upwards.
2. Ergosterol.	,, ,,	Red rapidly changing to violet.	Blue, changing rapidly to green and extending upwards.
3. Artostenone.	Yellow.	Yellow → red.	Red → violet → yellow.
4. Vitamin D (Radiostol B.D.H.)	Greenish yellow → orange.	Violet changing rapidly to brown.	Blue, immediately changing to green.
5. Cholic acid.	Yellow.	After about 20 seconds a fine blue ring appears which turns to violet in 2-3 minutes and becomes carmine red on long standing.	No blue ring or colour.
6. Desoxycholic acid.	Yellow (faint).	Brilliant violet ring after about 20 seconds.	No blue ring or colour.
7. Sodium glycocholate.	Yellow (faint).	Yellow → violet.	Blue → greenish yellow.
8. Sodium Taurocholate.	Yellow.	Yellow → violet.	Blue → greenish yellow.
9. Œstrogen from pregnancy urine.	Yellow.	Red → violet.	Violet → blue.

Attempts were next made to find out the minimum concentration of the steroid necessary for the development of the coloured rings. With cholesterol, it was observed that 0.5 mg. or more per cc. produced prominent rings, and the entire upper layer became distinctly blue in 1 minute. With 0.1 mg. also both the rings were found to be prominent. With 0.05 mg. the violet ring was prominent but the upper blue one was faint. With a concentration of 0.02 mg. only the violet ring made its appearance, and with 0.01 mg. no ring was detectable. Table II indicates results.

TABLE II

No. of solution.	Concentration (i.e. amount per cc.)	Observation.
1	2 mg.	The coloured rings are very prominent and the upper layer becomes distinctly blue in 1 minute.
2	1 mg.	
3	0.5 mg.	
4	0.2 mg.	The reaction is prominent giving 2 rings, (violet and blue).
5	0.1 mg.	
6	0.05 mg.	Violet ring prominent but blue ring is very faint. No upper blue layer on keeping.
7	0.02 mg.	Only a very faint violet ring is detectable, no blue ring or layer.
8	0.01 mg.	No ring observed.

Rosenheim and Callow (1) used an equal volume of 25% mercuric acetate in nitric acid as their reagent, but in this reaction it has been found that only 1 drop of even 1% solution of mercuric acetate in glacial acetic acid is quite sufficient and gives very satisfactory result.

The effect of the concentration of mercuric acetate on the depth and nature of the coloured rings is given in Table III.

TABLE III

Cholesterol used in each case is 0.1 mg., dissolved in 1 cc. of glacial acetic acid.

Concentration of mercuric acetate solution.	Amount of mercuric acetate solution used.	Observation.
Saturated	1 cc. added to 1 cc. sterol solution.	Violet and blue upper ring and no blue upper layer, which is very turbid.
,,	10 drops.	Violet and blue rings ; faintly blue upper layer.
,,	5 drops.	Very prominent rings (red, violet and blue, one above the other) and blue upper layer.
,,	1 drop.	Very prominent rings (red, violet and blue, one above the other) and blue upper layer.
5% solution	,, ,,	Do. Do. Do.
2% ,,	,, ,,	Do. Do. Do.
1% ,,	,, ,,	Do. Do. Do.
0.5% ,,	,, ,,	Coloured rings not so prominent.
0.2% ,,	,, ,,	Coloured rings not distinct.
0.1% ,,	,, ,,	Do. Do. Do.

SUMMARY

A new sensitive colour reaction for steroids has been developed.

REFERENCE

1.   ROSENHEIM AND CALLOW (1931), *Biochem. J.*, **25**: 74.

THE UTILISATION OF CALCIUM FROM GREEN LEAFY VEGETABLES

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A good deal of work has been done on the utilisation of calcium from vegetables. Mc. Cluggage and Mendel (1) found the calcium of carrot almost as well utilised as that of milk, the carrots furnishing half or more of the total calcium of a mixed diet. Blatherwick and Long (2) likewise concluded that the calcium as well as phosphorus of vegetables was satisfactorily utilised in meeting the maintenance requirements of healthy young women. Sherman and Hawley (3) found that the retention of calcium was more variable but less favourable when half of the milk of the diet was replaced by a carefully prepared mixtures of vegetables which provided the same total calcium intake. Bloom (4) found that the storage of calcium in 2 months old rats was less when spinach supplied 10% of the calcium of the diet than when spinach ash corresponding to an equal amount of spinach was fed.

Edelstein and co-workers (5) found that spinach-feeding lowered the calcium retention slightly, while the feeding of carrots produced no such change in the calcium balance. Schultz, Morse and Oldham (6) on feeding dried spinach to babies also reported a low utilisation of its calcium, not attributable to the cellulose. From experiments with adults Mellon, Johnson and Darby (7) found good utilisation of the calcium of leaf lettuce.

The literature cited above indicates that the calcium of spinach is less well utilised than that of carrots, lettuce and some other vegetables. Fincke and Sherman (8) have clearly shown that the poor utilisation of calcium in spinach is due to its high oxalic acid content. The work of Tisdall and Drake (9) shows that the calcium of spinach is not available and its presence in a diet makes a portion of the calcium from the other components of the diet also unavailable. The unavailability of calcium in part is due to the oxalic acid content.

Kung, Yeh and Adolph (10) report a "calcium retention co-efficient" of 15 to 21% for spinach. Of the twelve Chinese vegetables studied they find that, with the exception of spinach and soyabean sprouts, all show

calcium retention values comparable to that obtained with skim milk powder in the control diet. The low calcium retention value shown for spinach parallels its high oxalate content.

Speirs (11) and later Kohman (12) conclude from their work on rats that spinach not only supplies no available calcium but renders a considerable part of calcium of the other foods unavailable. Kohman finds that vegetables containing negligible oxalates produce excellent animals that deposit four times as much calcium per unit body-weight as those receiving spinach, which was found to contain 10% oxalic acid (dry basis).

The normal oxalate content of the blood is not raised by the consumption of spinach and other oxalate-containing vegetables (13), but the normal oxalate excretion in the urine is considerably increased in both rabbits (14), and in man (15).

Some green leafy vegetables have been examined to see how far they will be able to supplement a rice diet poor in calcium. *Amaranthus gangeticus* widely used in South India has been studied in detail.

EXPERIMENTAL

The edible portions of the vegetables were cut, thoroughly washed to free them from adhering mud, and dried in an air-oven (60°). They were then finely ground and sampled for analysis. The calcium and oxalic acid in the various leaves have been determined according to the methods of Mc. Cruden (16) and Arbenz (17) respectively.

The moisture, calcium, anhydrous oxalic acid, the calcium released by peptic digestion (Horwitt, Cowgill and Mendel, 18) and the ratio calcium: oxalic acid for some green leafy vegetables are given in Table I.

TABLE I

Name of the leafy vegetables	Moisture %	Calcium mg. %	Anhydrous oxalic acid mg. %	In vitro Digestibility % calcium released.	Calcium: Oxalic acid.
1. *Amaranthus gangeticus*	9.4	2500	8688	34	0.28
2. *A. inamœnus*	6.3	2753	8455	20	0.33
3. *A. mangostanous*	8.4	2247	7859˙	13	0.29
4. *Atriplex hortensis*	7.7	1000	11260	17	0.088
5. *Sesbania grandiflora*	8.4	2950	377	92	—
6. *Trigonella fœnum græcum*	13.4	1000	trace	74	—
7. *Hibiscus Sabdariffa*	7.7	1726	1980	71	0.87
8. ,, ,, (sour variety)	8.1	1937	1397	80	1.0
9. Lucerne	9.6	1954	trace	—	—
10. Spinach (Bangalore) 1.	5.7	1428	12150	12	0.12
11. ,, ,, 2.	5.5	773	10580	13	0.473
12. ,, ,, 3.	7.0	901	10080	26	0.089
13. *Piper betle*	15.3	877	2322	—	0.38
14. *Basella alba*	6.5	1688	7079	25	0.24

Growth Experiments.—In order to assess qualitatively how (*i*) a rice diet (poor in calcium), (*ii*) *Amaranthus gangeticus* diet (rich in calcium and oxalic acid) and (*iii*) a suitable combination of rice and *A. gangeticus* diet would affect the growth of young rats, three sets of experiments with the diets D_1, D_2 and D_3 (Table V) were started.

TABLE II
Materials used.

Description of the material.	Calcium. (mg. %)	Phosphorus. (mg. %)
1. Kahlbaum's egg albumin	74.2	81.7
2. Brown and Polson's patent corn flour	14.9	15.2
3. Cowlac skim-milk powder	1387	1000
4. Co. 9 raw (wooden hulled) rice	12.7	351
5. Vitamin B_1 solution (Betaxin tablets)	—	—
6. Marmite	—	—
7. Lois De Jongh's cod liver oil	—	—
8. Diamond sugar (big crystals)	—	—
9. Butter purchased locally	—	—

Co. 9 raw rice (wooden hulled) kindly supplied by the Paddy Specialist to the Government of Madras was used throughout this investigation. Avellar De Laureiro's (18) simple salt mixture (omitting calcium phosphate) was used.

Breeding and nursing rats were fed on the following stock diet with supplements of milk.

Stock diet.—Wheat (100 parts), casein (50 parts), starch (50 parts), linseed (30 parts), salt (4.5 parts), bone meal (4.5 parts), carrot (90 parts), butter (90 parts) and cod liver oil (15 parts).

Procedure.—Six young albino rats weighing 50-65 g. were kept in growth cages and fed the basal diet (Table IV, column 1) for a period of three days. Then they were changed over to the experimental diets (D_1, D_2 and D_3) Table IV for a period of 7 weeks. Food and water were given *ad libitum* and both renewed daily. Each rat was given a daily supplement of 1 cc. vitamin B_1 solution (5 I.U.), 5 drops cod liver oil and 0.3 g. marmite. The rats were weighed every week.

The growth of rats on diets D_1, D_2 and D_3 are given in Table V and the average weekly weights on these diets are given below.

TABLE III

Diet.	Initial wt.	Average weekly weights. (g.)							Average wt. increase per rat per week.
		1	2	3	4	5	6	7	
D_1	60 g.	70	79	88	90	—	—	—	—
D_2	59	69	75	83	90	92	88	84	3.6 g.
D_3	62	76	88	102	106	114	121	132	10.0

TABLE V
Growth of rats on diets.

Diet.		Weight. (g.)						Duration of the experiment. (Days.)
D₁	Rat No.	1 M*	2 M	3 M	4 F*	5 F	6 F	56
	Initial	60	58	57	59	63	62	
	Final	died	died	88	110	115	died	
D₂	Rat No.	7 F	8 M	9 M	10 M	11 F		49
	Initial	59	60	54	59	57		
	Final	71	92	78	91	77		
D₃	Rat No.	12 F	13 M	14 M	15 M	16 F		49
	Initial	64	62	63	62	60		
	Final	125	132	139	137	125		
D₄	Rat No.	26 M	27 F	28 M				32
	Initial	29	29	29				
	Final	48	died	died				
D₅	Rat No.	29 M	30 F	31 M				32
	Initial	31	30	30				
	Final	112	102	94				
D₆	Rat No.	37 M	38 M	39 F				32
	Initial	38	37	37				
	Final	39	37	37				
D₇	Rat No.	40 F	41 F	42 F				32
	Initial	34	34	34				
	Final	58	56	50				
D₈	Rat No.	32 M	33 M	34 M	35 M	36 F		35
	Initial	65	62	56	50	58		
	Final	109	106	102	101	118		
D₉	Rat No.	17 F	18 M	19 M				32
	Initial	36	33	35				
	Final	139	129	163				
D₁₀	Rat No.	20 F	21 F	22 M				32
	Initial	47	46	49				
	Final	117	118	128				
D₁₁	Rat No.	23 M	24 F	25 F				32
	Initial	49	43	42				
	Final	157	118	97				

*M = Male, F = Female.

In these experiments the procedure adopted was essentially that developed by Fincke and Sherman (8).

1 cc. of vitamin B_1 solution (5 I.U.), 5 drops of cod liver oil and 0.3 g. marmite were given as supplements with daily ration of each rat.

TABLE VI

Availability of calcium in diets D₄, D₅, D₆, D₇.

Diet.	Rat no.	Ca in body at 60 days or at death.	Ca in body at 28 days.	Ca ingested.	Ca retained.	Ca availability.
D₄	26 M	232.6 mg.	245.0 mg.	624.4 mg.	− 12.4 mg.	—
,,	27 F	194.7	,,	592.0	− 50.3	—
,,	28 M	246.0	,,	480.0	+ 1.0	—
D₅	29 M	404.0	245.0	1316.0	159.0	12.1%
,,	30 F	371.2	,,	1147.0	126.2	11.0
,,	31 M	359.0	,,	1142.0	114.0	10.0
D₆	37 M	301.1	269.9	169.7	31.22	18.4
,,	38 M	307.3	,,	192.4	37.40	19.5
,,	39 F	307.8	,,	200.1	37.9	19.0
						Average 18%
D₇	40 F	389.9	269.9	244.1	120.0	49.2
,,	41 F	403.6	,,	274.8	133.7	49.2
,,	42 F	376.2	,,	255.6	106.3	41.6
						Average 47%

High calcium level (D₄ and D₅).—Two out of three rats on diet D₄ died before the experimental period of 32 days while the surviving rat increased by 19 g. and practically retained no calcium. On the contrary, all the three rats on diet (D₅) flourished well retaining about 11% calcium on the average even at a high calcium level of intake. The average increase in the body weight was 73 g. Fig. 2 gives the growth curves of the rats on the two diets (D₄ and D₅).

Sub-optimum calcium level (D₆ and D₇).—The rats on diet D₆ maintained their initial weight and retained 18% calcium on the average. While the rats on D₇ increased by 20 g. (average) and retained 47% calcium on the average.

In order to demonstrate that the excess oxalic acid present in *A. gangeticus* renders at least a part of the usually available calcium of other foods unavailable, the following experiments were carried out.
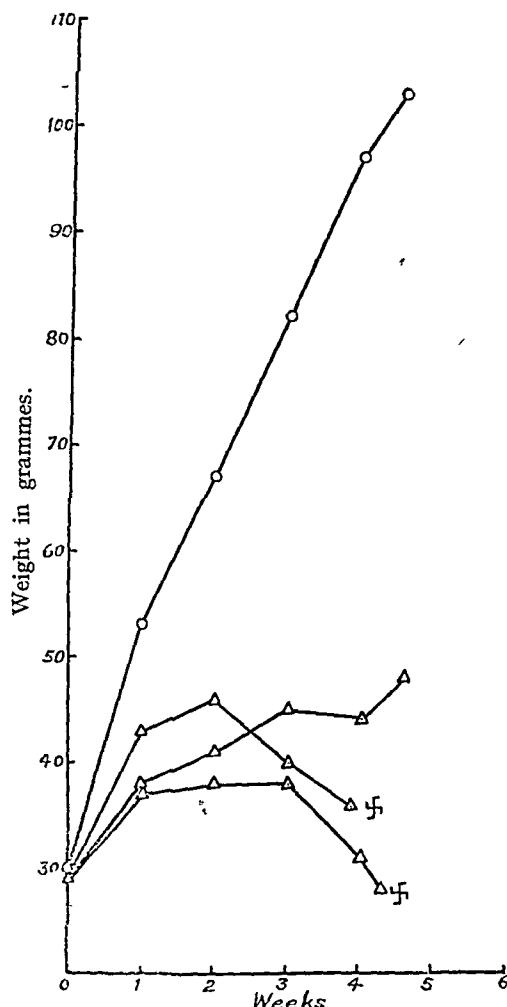
This photograph shows the differences in growth of the rats on diets, D_4 and D_5.

FIG. 2
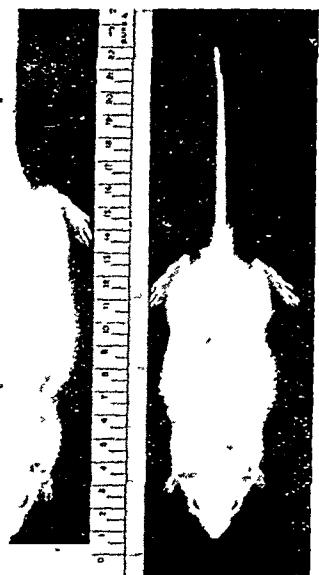
GROWTH CURVES

△———△ Individual Growth curves of rats on Amaranthus-gangeticus diet (D_4)

⊙———⊙ Average weight increase in rats on Amaranthus-gangeticus + rice diet (D_5).

Availability of calcium in diets (*i*) skim-milk, D_9 (*ii*) skim-milk and *A. gangeticus*, D_{10}, and (*iii*) skim-milk and *A. gangeticus*, D_{11} was carried out according to the technique of Fincke and Sherman (8). The experimental rats received their usual supplements of vitamins A, D, B_1 and B_2 along with their daily ration.

The composition, the growth of rats and the calcium availability of the diets (D_9, D_{10} and D_{11}) are given in Tables VII, V and VIII respectively.

TABLE VII
Composition of the diets.

Ingredients.	Skim-milk (control) (D$_9$)	A. gangeticus (D$_{10}$)	A. gangeticus and rice (D$_{11}$)
Whole wheat	63.7	63.7	63.7
Butter fat	10.7	10.0	10.4
Skim-milk powder	22.0	11.0	11.0
Corn flour	3.0	8.0	—
Sodium chloride	1.3	1.3	1.3
Amaranthus gangeticus	—	6.0*	6.0*
Rice	—	—	8.0
Moisture %	9.2	10.0	8.8
Calcium mg. %	332.0	335.0	335.0
Phosphorus mg. %	412.0	342.0	370.0

* 6 Parts of *A. gangeticus* contribute the same quantity of calcium as 11 parts of skim-milk.

TABLE VIII
Availability of calcium in diets D$_9$, D$_{10}$, and D$_{11}$

Diet.	Rat no.	Ca in body at 60 days.	Ca in body at 28 days.	Ca retained.	Ca ingested.	Ca availability.	
D$_9$	17 F	1098 mg.	262 mg.	836 mg.	836.4 mg.	100%	
,,	18 F	1009	,,	847	941.0	90.0	
,,	19 M	1129	,,	867	934.6	92.7	..
						Average 94%	
D$_{10}$	20 F	737	363	374	1018	36.8	
,,	21 F	703	,,	340	1035	32.9	
,,	22 M	720	,,	357	1051	34.0	
						Average 35%	
D$_{11}$	23 M	910	364	541	1021	53.5	
,,	24 F	708	331	377	904	41.7	
,,	25 F	666	331	335	733	45.7	
						Average 47%	

The percentages of calcium availability (Table VIII) of the diets D$_9$, D$_{10}$ and D$_{11}$ are 94, 35 and 47 respectively. The figures indicate that the calcium of *A. gangeticus* is not only unavailable but renders a part of the skim-milk calcium also unavailable. This can be prevented by the inclusion of rice in such a diet.

5

TABLE IX

Availability of calcium in linseed diet.

Rat No.	Calcium ingested.	Calcium excreted			Calcium retained (mg.)	Calcium availability. (%)
		Urine (mg.)	Fæces (mg.)	Total (mg.)		
First week						
32 M	119.5 mg.	7.7	25.2	32.9	86.6	72.5
33 M	90.4	2.0	13.4	15.4	75.0	83.0
34 M	68.7	4.2	11.7	15.9	52.8	76.9
35 M	91.0	2.4	10.1 ⁻	12.5	78.5	86.3
36 F	87.4	3.1	29.1	32.2	55.2	63.2
Second week						
32 M	123.0	4.2	19.5	23.7	99.3	80.7
33 M	124.0	2.4	23.7	26.1	97.9	78.8
34 M	84.7	9.2	14.6	23.8	60.9	71.9
35 M	102 3	5.8	7.2	13.0	89.3	87.2
36 F	107.6	4.4	9.3	13.7	93.9	87.2
Third week						
32 M	140.2	4.5	42.8	47.3	92.9	66.3
33 M	132.1	1.9	27.9	29.8	102.3	77.4
34 M	118.0	7.4	20.8	28.2	89.8	76.1
35 M	116.5	1.2	36.0	37.2	79.3	68.1
36 F	136 6	2.5	32.9	35.4	101.2	74.1
Fourth week						
32 M	145.5	3.9	34.1	38.0	107.5	73.9
33 M	122.9	1.8	18.8	20.6	102.3	83.2
34 M	165.9	7 1	22.7	29.8	136.1	82.0
35 M	162.2	1.1	51.0	52.1	100.7	65.8
36 F	164.7	1.8	33.6	35.4	129.3	78.5
Fifth week						
32 M	134.4	5.9	59.0	64.9	69.5	51.7
33 M	157.3	5.0	54.3	59.3	98.0	62.3
34 M	149 6	5.7	37.3	43.0	106.6	71.3
35 M	147.3	4.3	55.1	59.4	87.9	59.7
36 F	166.6	4.1	72.0	76.1	90.5	54.3
Average	125.9	4.1	30.5	34.6	91.3	73.0

The non-availability of the calcium of *Amaranthus gangeticus* is attributed to the presence of oxalic acid in it. With a view to investigate how far the calcium of green leafy vegetables with traces or no oxalic acid would be available, *Sesbania grandiflora* (412 mg. % anhydrous oxalic acid and 3231 mg. % calcium, both expressed on air-dry material) was chosen. The

calcium availability of this green leafy vegetable was carried out by the metabolic method of Henry and Kon (20).

Procedure.—Five albino rats weighing 50-65 g. were placed in individual metabolic cages on a basal diet (free from calcium ; see Table IV, column 1) for a period of three days and then on the experimental diet D_8 (Table IV) for a period of 5 weeks. A careful record of the food consumed was kept. Distilled water and daily supplements of 1 cc. of vitamin B_1 solution (5 I.U.), 5 drops of cod liver oil and 0.3 g. marmite were given. Weekly collections of fæces and urine were ashed separately and analysed for calcium. The composition, the growth of rats on this diet and the availability of calcium are given in Tables IV, V and IX respectively.

It is seen from Table IX that the average availability of calcium of this diet is 73% even at a high calcium level of intake.

DISCUSSION OF RESULTS

Sesbania grandiflora, Trigonella fœnum græcum and Lucerne contain negligible quantities of oxalic acid (Table I) and the first one has the highest calcium content (3231 mg. %) of all the greens investigated. The other leaves contain good amounts of oxalic acid, the two varieties of *Hibiscus Sabdariffa* containing relatively small quantities. The ratio of calcium to oxalic acid gives us an idea of the relative calcium availabilities. The higher the ratio, the better the availability. The ratio is least in *Atriplex hortensis* and spinach varieties ; next come in order the *Amaranthus* varieties and *Basella alba* both of which are commonly used in South India. The amount of calcium released by the *in vitro* peptic digestion gives a rough indication of the calcium availability. In our preliminary experiments we found that 0.1 N-hydrochloric acid alone dissolves almost as much of calcium as HCl-pepsin does. This observation was made previously by Horwitt, Cowgill and Mendel (18).

Before discussing the growth of rats on various diets in this investigation, it must be clearly borne in mind that the rats get enough protein, vitamins A, D and factors of B-complex from their daily ration. Therefore the difference in the growth responses of rats on the various diets cannot be attributed to the deficiency of any of the vitamin factors or protein.

The growth of rats on *Amaranthus gangeticus* and rice diets D_3 (10 g. per week ; Table III) is found to be better than that of rats on *A. gangeticus* diet D_2 (3.6 g. per week). This better growth could not be entirely attributed to the addition of rice since the diets were not identical. The difference being that diet D_2 contained 15% while diet D_3 had only 10% *A. gangeticus*.

The results of the calcium retention experiments with diets D_4, D_5, D_6 and D_7 done under strictly comparable conditions, conclusively prove the beneficial effect of rice even when the experiments are carried out at high and sub-optimal levels of calcium intake. In all the diets the quantity of calcium

contributed by *A. gangeticus* is 94% of the total. The decalcification and death in the rats on D_4 are not due to the rachitogenic action of corn flour because a daily supplement of 5 drops of cod liver oil are given to each and this effectively counteracts the anti-calcifying effects of the corn flour. The level of calcium in the diets (D_1 and D_5) is high (0.45%) and the calcium ingested is much more than that required for normal retention as given by Sherman and McCleod (21). This high level is the outcome of our trying to have a high oxalic acid content in the diet. At sub-optimal calcium level of intake, the utilisation of calcium from diets D_6 and D_7 is 18 and 47 respectively.

The *A. gangeticus* diets D_2 and D_4 are practically the same. All the rats on diet D_2 could live longer and keep up some weight while two rats on diet D_4 lost their body calcium and died within five weeks. As rats weighing 50-65 g. were used with diet D_2 and rats weighing 29 g. were used with diet D_4, it looks as though the older rats are able to utilise the calcium to some extent from calcium oxalate and excrete the oxalic acid, or, are able to oxidise the oxalic acid partially and take time to be poisoned. In this connection it is necessary to mention the significant observation of Kohman (23) that the oxalic acid recovery in the excreta (urine and fæces) becomes less as the rats grow older.

Results of the retention studies of calcium in skim-milk diets (Table VIII) show that 94% of the calcium of skim-milk is available. This figure is in accordance with the values obtained by various workers. Henry and Kon (20) got a retention figure of 98% in the presence of extra phosphorus and sub-optimal quantities of calcium. When 50% of the skim-milk calcium is replaced by *A. gangeticus* calcium, we expect a calcium availability of 47% even if all the calcium of the latter is completely unavailable. But the average calcium availability in diet D_{10} is only 35% showing that the oxalic acid present in *A. gangeticus* renders some skim-milk calcium also unavailable. This observation is in agreement with the results of Fincke and Sherman (8), Speirs (11) and Kohman (12), on spinach. The addition of rice in diet D_{11} instead of corn flour raises the availability figure from 35 to 47%. This shows that introduction of rice prevents the skim-milk calcium from being rendered unavailable by the oxalic acid present in the *A. gangeticus*.

The calcium availability of *Sesbania grandiflora* leaves containing very little oxalic acid (0.41%) and calcium (3.2%) has been estimated by the metabolic method of Henry and Kon (20). The calcium availability is 73% (average) even at a high calcium level (0.32%) of intake. In the metabolic experiments the level of calcium is of great importance. The calcium ingested should be sub-optimal such that the maximum retention figure can be obtained. In the present work the food consumption of rats on the linseed diet is low perhaps due to the bitter principle which the leaf contains.

Hence the calcium ingested for the first two weeks is below the optimum quantity for normal maintenance. In the last three weeks the rate of growth is less and the calcium required is naturally low and the ingestion is more. The availability figures for the fifth week are lower than those of the other four weeks. Throughout the experimental period the calcium excreted in the urine is much less than the calcium excreted in the fæces.

The observations that leaves containing no oxalic acid are good sources of available calcium and the unavailable calcium of greens rich in oxalic acid is rendered available to a great extent by the addition of rice, are of great importance from the point of view of the poor South Indian diet containing a preponderance of rice.

In all the experiments only Co. 9 raw rice (hulled in a wooden huller) has been used. It remains to be investigated whether milled rice would produce the same effect. The mechanism of the detoxication of oxalic acid by the inclusion of hand-pounded rice in the *A. gangeticus* diet is also a future line of work.

Summary

Some green leafy vegetables commonly used in South India have been analysed for their calcium and oxalic acid contents.

It has been shown that a diet containing *A. gangeticus* or rice alone cannot induce growth in rats while a combination of the two does.

The calcium of *Amaranthus gangeticus* is unavailable and produces decalcification and death due to the presence of excess oxalic acid in it. While the inclusion of rice in the *A. gangeticus* diet renders at least part of its calcium available and hence produces better growth and retention of calcium in rats.

The oxalic acid present in *A. gangeticus* renders also a part of the usually available calcium of skim-milk, unavailable.

The calcium of *Sesbania grandiflora* (0.4% oxalic acid) is available to the extent of 73% even at a high level (0.32%) of calcium intake.

It can finally be concluded from the results of this investigation that green leafy vegetables (irrespective of their oxalic acid content) rich in calcium can be recommended as suitable supplements for a rice diet poor in calcium.

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A SENSITIVE CHEMICAL TEST FOR THE DETECTION OF ARGEMONE OIL. PART II. THE SPECIFICITY OF THE TEST

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In a recent communication the present author (1) has reported on the development of a simple chemical test for the detection of argemone oil, alone or in mixture with mustard oil. In this connection he has also pointed out that the nitric acid test, so extensively used by Lal *et al* (2) for this purpose, is far from satisfactory. In fact some dropsy-positive samples of mustard oil (which appeared to contain a fairly good amount of argemone oil as tested by the nitric acid method) failed to give the ferric chloride test proposed by the author showing that those mustard oil samples did not contain argemone oil even in a concentration of 0.75%. The present investigation was undertaken with a view to study the specificity of the ferric chloride test for argemone oil.

EXPERIMENTAL

The ferric chloride test and the nitric acid test were both applied to a large number of vegetable oils. The observations are recorded in Table I.

It appears from the observations reported in the accompanying table that out of fourteen vegetable oils examined nine samples were positive to the nitric acid test but none of them really gave positive ferric chloride test for argemone oil. The list of oils examined includes a large number of possible adulterants of mustard oil and under these circumstances any attempt to detect argemone oil in mustard oil with the help of the nitric acid test alone will be very misleading. However, this question does not come in when the ferric chloride test is applied. This test is, therefore, much more specific than the nitric acid test.

TABLE I

Sample of oil.	Mode of extraction.	Source of supply.	Nitric acid test: colour of the acid layer.	Ferric chloride test and nature of the precipitate, if any.
Castor oil	Expeller	H. B. Technological Institute, Cawnpore	Colourless	No orange-red fibrous crystalline precipitate, i.e., negative argemone test.
Cocoanut oil	Ghani	Do	Do	Negative
Groundnut oil	Do	Do	Do	Do
Linseed oil	Expeller	Do	Light yellow	Do (slight grayish amorphous precipitate)
Mahua oil	Ghani	Do	Deep orange red	Negative
Niger seed oil	Do	Do	Deep reddish brown	Do
Poppy seed oil	Do	Do	Colourless	Do
Safflower seed oil	Do	Do	Light yellow	Do
Sesame oil	Do	Do	Orange	Do (slight gray or black precipitate)
Cotton seed oil	Expeller	Modi Vanaspati Mfg., Co., Begamabad.	Colourless	Negative
Olive oil	...	Market	Light orange	Do
Jute seed oil	Solvent	Dr. N. K. Sen	Deep red	Do
Tamarind oil	Do	Mr. M. C. Malakar	Light yellow	Do
Radish oil	Do	Author	Do	Negative

SUMMARY

Fourteen samples of vegetable oil, many of which are adulterants of mustard oil, were tested both by the ferric chloride and the nitric acid tests. Nine of them were positive to the nitric acid test but all of them were negative to the ferric chloride test for argemone oil, showing that the latter test is fairly specific for the purpose.

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STUDIES IN VITAMIN A METABOLISM. PART I. EFFECT
OF THE INCLUSION OF COCONUT CAKE IN THE BASAL
DIET ON THE UTILISATION OF CAROTENE BY
VITAMIN A-DEFICIENT RATS

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While vitamin A is almost completely assimilated at ordinary levels of intake, the absorption[1] and utilisation of the provitamins are extremely variable and seem to be influenced by a variety of factors (1—5). The effect of the nature and amount of dietary fat, though considerable, appears to have little physiological significance (6—9). It may be mainly a kind of "solvent effect", the highest absorption of carotene (nearly 85%) being obtained when administered to the animals as a solution in a suitable oil. Complete absorption of the ingested carotene, however, has not been reported so far.

Recently, Wilkinson and co-workers, (10, 11) made the interesting observation that the inclusion of 30% of coconut cake meal in the basal diet of vitamin A-deficient rats profoundly affected their capacity to absorb and metabolise carotene and vitamin A. According to them, the animals on such a diet showed better growth response, low hepatic reserves of vitamin A and no fæcal excretion of carotenoid. The controls, on the other hand, excreted a considerable amount of carotene, possessed high reserves of probably unutilised vitamin A and showed very inferior growths. They adduced further evidence to suggest that coconut cake contains a "factor", probably protein by nature, which is essential for the conversion of carotene into vitamin A and its subsequent utilisation. In view of the fundamental importance of these findings in the study of vitamin A metabolism, it was thought desirable to independently confirm the results and continue the line of investigation.

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1. The term "absorption", as used in these papers, denotes the difference between the amount of carotene ingested and the amount recovered in the fæces ; therefore it includes also any carotene that might have undergone destruction in the physiological system or in the fæces after excretion.

Experiments were, therefore, carried out seeking information on three points: (*a*) Does the inclusion of 30% of coconut-cake in the basal diet produce cent. per cent. absorption of carotene? (*b*) Do the animals on such a diet show better growth than the controls? (*c*) Do the control animals possess comparatively large reserves of vitamin A which they are unable to utilise?

While this investigation was in progress, Bacharach (12) published the results of his studies on the use of various basal diets for vitamin A assays. Based only on the growth responses, he reported that the inclusion of coconut cake rendered the basal diets unsuitable for vitamin A assays. It was, however, felt worthwhile to investigate the other aspects of the question and therefore, experiments on hand were continued.

EXPERIMENTAL

Young albino rats were employed as experimental animals. The diet of the mothers was restricted in order that the young ones might not have large reserves of vitamin A. When the animals were about four weeks old, they were placed on the vitamin A-free basal diet No. 1 whose composition is shown in Table I. By about the fifth week, some of the animals showed

TABLE I

Composition of the Vitamin A-free Basal Diets.[1]

		Diet No.					
	1	2A	2B	3A	3B	4A	4B
Extracted casein[2] ...	18	15	10	15	12	—	—
Heated casein[3] ...	—	—	—	—	—	15	9
Polished rice flour ...	59	70	48	63	45	—	—
Rice starch ...	—	—	—	—	—	63	48
Coconut cake[4] ...	—	—	30	—	30	—	30
Tomco's coconut oil ...	10	3	—	4	—	4	—
Dried Brewer's Yeast ...	8	8	8	8	8	8	8
Salt mixture[5] ...	5	4	4	5	5	5	5
Agar agar ...	—	—	—	5	—	5	—
Vitamin D supplement ...	0.35 microgram of calciferol in arachis oil per rat per week.						

1. The diets have been planned on the assumption that coconut cake contains 20% of protein and 12% of oil and polished rice contains 6% of protein.

2. Polson's "Lactic casein" was refluxed with 96% alcohol for two hours and the alcohol was pressed out in a tincture press. This treatment was repeated once more and the pressed casein was dried in a stream of air at 45°.

3. Casein extracted as in (2) and dried at 100° for 48 hours.

4. Kindly supplied by the Tata Oil Mills Ltd., to whom the author's thanks are due.

5. Salt mixture prepared according to Hawk and Oser (13).

symptoms of xerophthalmia but all ceased to grow or slightly declined in weight. Then, they were divided into two groups, employing litter-mate pairing of rats of the same sex and approximately the same body-weight, and were transferred to the experimental diets, feeding being *ad libitum*. The only difference between the diets consumed by the paired rats was the presence of 30% of coconut cake meal in diet B and the complete absence of it in diet A (*vide* Table I). Carotene (B.D.H.)[2] dissolved in oil was fed directly, by means of a dropper, into the mouth of each animal daily ; in all the experiments to be described in this paper, the supplement was 24 micrograms of carotene per rat per day. Since the B.D.H. carotene contains some ∝-carotene also, the supplement should be considered to be slightly less than that used by Wilkinson and co-workers (*loc. cit.*).

The carotene solution in coconut oil (Tomco's) was prepared by dissolving it first in peroxide-free ether, adding it along with a drop of an alcoholic solution of hydroquinone to the required amount of oil and finally removing the solvents under reduced pressure at a temperature not exceeding 45°. The solution was put in a brown glass bottle and preserved in the refrigerator ; the concentration of the solution was checked twice a week. It was subsequently observed that carotene was more stable in arachis oil than in coconut oil and therefore, arachis oil was used as the solvent for experiments III and IV.

The animals were weighed at weekly intervals during the three-week experimental period.

At the outset, the fæces of the rats were collected thrice a week and carotene excretion determined for each rat individually. This was found to be laborious without any additional advantage and so, during later stages of the experiment, the fæces were collected twice a week and determinations were made on weighed samples of the fæces of each group pooled together.

At the end of the three-week test period, the animals were starved overnight and were killed by a knock on the head. The livers were excised out, minced and placed in 8% potassium hydroxide solution for vitamin A determination.

Methods.—For the determination of carotene, a weighed sample (3—5 g.) of fæces was ground to a fine powder and refluxed for 10 minutes with 30 cc. of absolute alcohol. After centrifuging, the alcohol was decanted off and filtered through cotton wool. The residue was refluxed with a further 20 cc. of alcohol and the extract centrifuged. The residue was then extracted in the cold with two 15 cc. portions of petroleum ether (b.p. 40—60°) followed by 10 cc. of absolute alcohol. A small quantity of water was added to the combined extracts and the pigments were taken up in petroleum ether,

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2. The author is grateful to Dr. Bashir Ahmad for the gift of 100 mg. of this substance.

the aqueous alcoholic layer being extracted twice or thrice more with petroleum ether. The combined extracts were washed repeatedly with water, concentrated to a small volume (about 10 cc.) in an atmosphere of CO_2 and refluxed with 10 cc. of 5% alcoholic potash for 10 minutes. The unsaponifiable matter was taken up in petroleum ether and washed with water. A measured quantity of anhydrous methanol, approximately equal to the volume of the petroleum ether solution, was added and after shaking, enough water was added to the mixture to bring the alcohol concentration to 85%. The methanolic layer was drawn off and the treatment was repeated, making the final concentration of methanol 90%. The epiphasic layer was repeatedly washed with water and dried over anhydrous sodium sulphate. The solution was filtered over a wad of cotton wool covered with anhydrous sodium sulphate, made up to a suitable volume and the carotene content was determined according to Ferguson (14) by colorimetric comparison against 0.1% potassium dichromate solution.

The vitamin A content of each liver was estimated by the Carr-Price reaction employing the experimental procedure described by Lease *et al* (15) ; the values were calculated according to Moore (16). Statistical examination of the results was according to Fisher (17).

Carotene excretion.—Kemmerer and Fraps (3) reported the presence of a non-carotene pigment in ,the fæces of rats and chickens which were not receiving any carotenoid supplement. On analysis, this was found to be less than half a microgram carotene-equivalent per rat per day (18) ; this being negligible, no correction was applied to the observed values.

Experiment I.—The results of the first experiment showed that a considerable amount of carotene was excreted by all the rats as can be seen from Table II. There was practically no difference in the total amounts of carotene excreted by the animals of either group whereas Gridgeman *et al* (11) reported that coconut cake effected a complete absorption of the carotene by virtue of the presence in it of an essential dietary "factor" in which Glaxo "physiological caseinate" and meat meal were found lacking.

Experiment II.—In the unavoidable circumstances of Glaxo "caseinate" being not available, two other sources of protein were tried on the next set of animals, to see if the carotene absorption would be substantially altered. In the first week, the diets contained commercial "light white casein" and in the two succeeding weeks it was replaced by dried mince-meat and extracted casein respectively. Agar agar (5%) was included in diet 3A in order to balance the crude fibre content of coconut cake ; the protein content of diet 3B, however, was slightly greater than that of diet 3A. The results of this experiment are presented in Table III.

The figures indicate a slightly better absorption by the animals consuming the coconut cake diet, particularly during the second and third weeks. While

this effect cannot be attributed to, any single factor, it should be observed that in neither of the groups was the carotene absorption quantitative.

TABLE II

Effect of the inclusion of coconut cake in the basal diet on the absorption of carotene by vitamin A-deficient rats (Experiment I).

Each group consisted of 2 male and 3 female rats. Diet No. 1 was used during the depletion period. Carotene supplement was 24 micrograms per rat per day.

	Micrograms of carotene excreted by the rats on	
	Diet 2A	Diet 2B
First week	290	306
Next 4 days	141	160
,, 4 ,,	127	160
,, 4 ,,	164	153
,, 3 ,,	114	86
Total excreted	836	865
Total administered	2520	2520
Per cent absorbed	67	66
Average growth response (g.)	31	30
Average hepatic stores of vitamin A (Moore Blue values)	166	150

TABLE III

Effect of different proteins and coconut cake on the absorption of carotene by vitamin A-deficient rats (Experiment II).

Each group consisted of 4 male and 2 female rats. The figures represent the total amounts of carotene excreted by the animals of each group.

	Protein supplied.	Animals on diet.	
		3A	3B
First week	L. W. casein	168 mg.	227 mg.
Second ,,	Dried mince-meat	493	392
Third ,,	Extracted casein	427	240
Total excreted		1088 mg.	859 mg.
Total administered		3024 mg	3024 mg.
Per cent absorbed		64	72
Average growth response (g.)		47	37
Average hepatic stores of vitamin A (Moore Blue values)		223	175

Experiment III.—In order to test the possibility of the extracted casein or the rice flour having supplied the postulated "factor" to the animals, experiments were conducted using Coleman's (rice) starch and heated casein in place of the rice flour and alcohol-extracted casein respectively (*vide* Table I). Whereas, in the earlier experiments, the animals were depleted of their vitamin A reserves on basal diet No. 1 and transferred to the experimental diets when deficiency was produced, the procedure was slightly altered to conform to that employed by Gridgeman *et al* (*loc. cit.*). The young rats were paired at the time of weaning and directly placed on diets 4A and 4B. When the growth plateau occurred, the usual carotene supplement was begun without any change in the basal diets. The results of this experiment, presented in Table IV, are in accord with those obtained in earlier experiments. It should further be noted that the animals on diet 4B possessed higher liver reserves of vitamin A while their growth-response was inferior to that of the diet 4A group.

Experiment IV.—In another experiment, the animals were divided into three groups at the end of the depletion period (diet 2A) and transferred to diets 4A, 2A and 3B respectively. The main features of these diets were: diet 4A contained pure rice starch and heated casein while the other two had rice flour and the usual extracted casein ; diet 3B contained 30% of coconut cake while the other two contained none. The results (*vide* Table IV) show that the replacement of rice starch by the rice flour produced an increase in the growth response, though the absorption of carotene appears to have been lowered. The figures, further, show that coconut cake had an adverse effect on the growth response.

TABLE IV

Influence of coconut cake feeding on the utilisation of carotene by vitamin A-deficient rats (Experiments III and IV).

Each group consisted of 4 male rats. The figures represent the averages for each group. Daily supplement was 24 micrograms of carotene per rat.

	Experiment III.		Experiment IV.		
	Rats on diet 4A.	Rats on diet 4B.	Rats on diet 4A.	Rats on diet 2A.	Rats on diet 3B.
Growth during depletion period of 34 days (g.)	71	60	—	—	—
Growth during 3-week experimental period (g.)	49	38	42	54	45
Hepatic stores of vitamin A (Moore Blue values)	69	106	43	48	37
Absorption of carotene (per cent)	85	73	84	75	82

The better growth obtained on rice flour is perhaps due to the protein of high biological value contained in it. But the evidence does not warrant any rôle in carotene metabolism being attributed to the rice-protein, on that account.

Growth response and vitamin A storage.—The amounts of vitamin A stored in the livers of the rats at the end of the 3-week test period and the corresponding growth-responses are shown in Table V. The results obtained from all the experiments have been combined, since the only difference between the diets of paired animals was the presence of 30% of coconut cake or the total absence of it. Thereby, it was possible to examine the results statistically and find the significance of the differences. It will be seen that, for the same amount of carotene supplement, the differences in growth response are highly significant ($p=0.01$) and that diet A is far superior to diet B containing coconut cake. The results also show that the animals on the A-diets possessed, on the average, 13.7 Blue values of vitamin A per liver more than the controls. This difference, however, is not statistically significant ($p=0.35$ approximately). It would thus appear that the inclusion of coconut cake in the basal diet has had, if at all, only a deleterious effect as judged by the growth and vitamin A storage. Summing up the results of the "absorption" experiments, (*vide* Tables II, III and IV) it may be said that the rats were excreting the same amount of carotene irrespective of the presence or absence of coconut cake in their diets.

In some experiments, the results of which are not reported here, it was observed that the use of a fresh sample of coconut cake obtained by pressing out the oil from dried copra in a hydraulic press, did not produce any noticeable differences in the values. Failure to confirm the results of Wilkinson and co-workers does not seem to be as much due to any essential differences in the composition of coconut cake from various sources as to possible contamination of their coconut cake with vitamin A or carotene. Their finding that palm kernel meal and acetone extracted herring roe contains the new "factor" suggests the same as a possible explanation.

DISCUSSION

Wilkinson and co-workers (*loc. cit.*) mention, however, that their observations refer to the deficiency of an essential protein "factor" in Glaxo "physiological caseinate" and meat-meal which were employed in their studies and that other caseins may not necessarily be lacking in it, though they had noticed no difference by using at least one more brand of casein (name not mentioned). Glaxo "physiological caseinate" was, unfortunately, not available in India and therefore, Polson's casein was employed in the experiments described. The possible argument that this casein might not have been deficient in the "factor" is ruled out by the fact that all the rats were excreting appreciable amounts of the ingested carotene.

TABLE V

*Effect of coconut cake feeding on growth response and
vitamin A storage.*

Three-week test on a supplement of 24 micrograms of carotene per rat
per day.   Values for each individual rat are given.

Expt. No.	Sex of the animal.	Growth response (g.) of the animals on diet.		Vitamin A stores (Moore Blue Values per liver) in the animals on diet.	
		A	B	A	B
I.	m	55	46	44	53
	f	14	16	209	253
	f	23	38	160	138
	m	.3	38	198	77
	f	18	12	220	231
II.	f	35	36	318	236
	f	53	32	240	208
	m	47	26	312	307
	m	58	51	185	163
	m	45	42	219	64
	m	42	36	64	69
III.	m	51	24	75	187
	m	45	32	78	85
	m	49	54	69	75
	m	52	41	54	79
IV.	m	64	52	40	40
	m	45	39	43	35
	m	55	48	20	32
	m	52	41	88	43
V.*	m	58	48		
	f	35	40		
	m	58	29		
	m	57	44		
	f	42	35		
	f	36	31		
	m	48	45		
	Average	45.4	37.3	138.7	125.0
	Mean difference	+7.8		+13.7	
	Standard deviation of differences	12.6		60.1	
	Standard error of differences	2.47		14.77	
	"t" =	3.16		0.94	

*Diets 2A and 2B were used in this experiment.
m = Male ; f = Female.

There has been a considerable amount of controversy over the choice of a type of casein suitable for incorporation into vitamin A-free basal diets. Coward and co-workers (19—21) found Glaxo "vitamin-free casein" to be unsuitable for vitamin assay work since it lacked a hitherto unknown "factor" necessary for the normal growth of the rat. They advocated the use of untreated "light white casein" (B.D.H.) for obtaining consistent and reliable results. Bacharach (22) on the other hand, could not find any differences in growth response when different types of casein were used in the basal diets. On the basis of biological experiments, Culhane (23) observed that "light white casein" contains vitamin A in sufficient amounts to prohibit its use in vitamin A-free diets while extracted and heated casein was found by her to be completely free from vitamin A. Maitra and Moore (24) actually estimated the vitamin A content of "light white casein" to be about 1 I.U. per g. and confirmed this finding by biological experiments. In their experience, double extraction with hot alcohol was the best method for completely removing vitamin A from the casein and this procedure has been adopted in this laboratory also.

Considering the fact that Coward *et al* (*loc. cit.*) observed the absence of their heat-labile growth "factor" in Glaxo "vitamin-free casein" and that Wilkinson and co-workers noticed the inadequacy of Glaxo "physiological caseinate" for the efficient utilisation of carotene and vitamin A, the question naturally arises whether both these "factors" are the same. Again, there is still another growth-stimulating factor "physin" whose existence in fresh ox-liver was postulated by Mapson (25). Further work, either confirming or extending the work on these "factors", is unfortunately lacking. It should, nevertheless, be admitted that the usually employed synthetic basal diets are far from the ideal (22) ; it is common experience in vitamin assay work to find that some of the experimental animals in which vitamin A-deficiency has been produced, fail to resume growth or show erratic growth when the diet is supplemented with vitamin A or carotene. Further work may reveal in these diets the deficiency of some "factor" essential for the growth of the rat. But, at present, there is no convincing evidence to suggest that any of these postulated "factors" has any direct rôle in vitamin A metabolism.

SUMMARY

Effect of the inclusion of coconut cake in the basal diet on the absorption and utilisation of carotene by vitamin A-deficient rats has been studied.

Using alcohol-extracted casein as the main source of protein, it was not possible to confirm the claims of Wilkinson and co-workers on the presence in coconut cake of a "factor" essential for vitamin A metabolism.

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For the same amount of carotene supplement (24 micrograms per rat per day), the animals consuming coconut cake gave growth-responses far inferior to those given by the controls while the differences in carotene excretion and hepatic reserves of vitamin A at the end of the three-week experimental period were not significant.

It would seem, therefore, that the inclusion of coconut cake has had, if at all, only a deleterious effect.

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VITAMIN A AND D CONTENTS OF CERTAIN FISH-LIVER OILS

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Although the vitamin A, B_1, B_2, C as well as nicotinic acid contents of a large number of fresh-water fishes of Bengal have been determined and reported in earlier publications (1—6), no systematic investigations have been carried out regarding the vitamin D values of the fish-liver oils. Our previous investigations, concerning the vitamin A values of the body and liver oils of a number of Bengal fish, indicate that the liver oils of some of these are considerably more potent in vitamin A than cod-liver oil, though not as rich as halibut liver oil, and the body fats are, of course, very much poorer than the liver oils. The results concerning the vitamin D content of the body and liver oils of a few varieties of Bengal fish have been reported by Basu and Sen-Gupta (7). But, as the number of available varieties, which commonly constitute important ingredients of the daily diet in Bengal, is very great, we considered it desirable to carry out some more investigation on the vitamin D content of the liver oils of Bengal fish. The vitamin A-content of these oils was also determined.

Shark-liver oil, which is richer than cod-liver oil in vitamin A is said to be poorer in vitamin D, although some more work on the subject seems to be necessary. It appears worthwhile to pursue systematic investigations on sea-fish like those obtained from the Bay of Bengal which might serve as rich sources of both vitamins A and D. Moreover a prolific sea-fish of this type, if discovered, would be industrially of greater value as its supply is likely to be steady and plentiful.

The present communication deals with the results of estimations of the vitamin A and D contents of liver oils of *dhain, vetki* and *sankhachur*, of which the last is a sea-fish. These were obtained during the months of September and October, 1941.

EXPERIMENTAL

Preparation of the oils.—The finely minced liver was ground up with excess of anhydrous sodium sulphate and extracted in a Soxhlet apparatus for 8—10 hours with ether. The ether was distilled off and finally the oils were dried.

The estimations of vitamins A and D for each kind of fish were carried out usually after pooling together the same quantity of oil, extracted from the livers of 4 to 5 specimens of the same variety of fish ranging from the maximum to the minimum sizes available in the market in the months of September and October.

Estimation of Vitamin A.—The vitamin A content of the samples was determined tintometrically, as described by Carr and Price (8). Estimations were also carried out with the unsaponifiable fraction of the oils (except *Sankhachur*), which were prepared by saponifying 1 g. of the crude oil according to the method recommended by the Medical Research Council (9). Table I gives the mean values of several consecutive readings. The figures in the last column were obtained by converting the Carr-Price values into international units by the following relation given by Heilbron, Gillam and Morton (10).

$$E^{1\%}_{1\ cm.}\ 1.0 = 12.5\ \text{Carr-Price.}$$

$$E^{1\%}_{1\ cm.}\ 1.0 = 2200\ \text{international units.}$$

TABLE I

Bengali name of fish.	Zoological name.	Carr-Price value of crude oil.	Carr-Price value of the non-saponifiable fraction expressed in terms of crude oil.	International units of vitamin A per g. of oil.
Sankhachur	...	2.20	...	387.2
Dhain liver oil	*Silonia Silundia*	5.6	3.2	563.2
Vetki liver oil	*Lates calcarifer*	3.8	2.6	457.6
Cod liver oil*	...	9.5	8.8	1548.8

*This sample of Squibb's cod-liver oil was tested for comparison.

Estimation of vitamin D by the prophylactic method.—The method adopted in this experiment was essentially the same as described by Chick, Korenchevsky and Roscoe (11) and followed by Key and Morgan (12).

Twenty albino rats, weighing between 35 and 45 g., were selected and divided into 5 groups, having 4 in each group. Each of the animals was

placed in a separate cage with a raised screen to prevent access to the excreta. The experiments were conducted in a room shielded against sunlight. The young rats were placed on a Steenbock's rachitogenic diet, No. 2965 (Steenbock and Black, 13) having the following composition:

Ground yellow maize 76%
Wheat gluten 20%
Calcium carbonate 3%
Sodium chloride 1%

The diet was given in the form of a paste mixed with a little water. Each of the animals of the 1st group received in addition to the basal rachitogenic diet, 0.2 unit of a standard source of vitamin D, per animal per day ; those of the 2nd group were kept as negative controls and the remaining 3rd, 4th and 5th groups were supplemented with 30, 40 and 70 mg. of *dhain, sankhachur* and *vetki* fish liver oils respectively per animal per day. The probable potency of each of these doses of the test materials was found to be of the order of 0.2 unit of vitamin D by a preliminary experiment, using the curative method (Bills, Massengale and Ball, 14) with a smaller number of animals. The feeding was continued for 35 days after which the animals

TABLE II
Vitamin D content of fish liver oils.

Sources of supplementary vitamin D.	No. of animals.	Dose of oil in mg.	Percentage of ash in bone.	Mean % of ash in bone.	International units of vitamin D per g. of oil.
Standard vitamin D solution	4	0.2 international unit.	46.25 51.15 41.36 49.00	46.35	...
Dhain-liver oil	4	30 mg.	36.63 47.68 45.70 46.67	44.22	6.67
Sankhachur-liver oil	4	40 mg.	40.82 47.50 42.56 49.69	43.62	5.00
Vetki-liver oil	4	70 mg.	44.12 39.70 43.31 49.77	44.22	2.86
Nil.	4	...	31.52 33.71 33.31 30.68	32.3	...

were killed. The femora of the rats were removed and freed as completely as possible from adhering tissues. Each bone was tied up in a piece of muslin and extracted with boiling alcohol and then with ether for 6—8 hours. The bones were then dried and weighed and the percentage of ash in the fat-extracted dry bone for each animal was determined after ashing. The results are given in Table II.

DISCUSSION AND SUMMARY

From Tables I and II it is clear that the vitamin A and D contents of the three different fish-liver oils (dhain, vetki and sankhachur) analysed are much less than that of the sample of cod-liver oil (Squibb's) whose vitamin A content in international units is of the order of 1500, and whose vitamin D content per g. of oil is 300 units as specified on the bottle. Table I also shows that the Carr-Price values of the crude oils are somewhat higher than those of the non-saponifiable fractions, owing probably to the presence of some chromogen other than vitamin A in the crude oil.

It is evident that none of the samples of the fish analysed can be taken as a good source of vitamin A or D, but of the three, Dhain is the most potent both with regard to vitamins A and D. Although sankhachur was investigated particularly as a sea-fish, it had really no advantage over the other fresh-water fishes as a source of vitamin A or D. It seems that a rich source of vitamin D has not yet been found among the liver oils of different fishes so far investigated in India.

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THE INTRADERMAL TEST AS AN INDEX OF VITAMIN C-
NUTRITION. PART III. THE RELATION BETWEEN
URINARY ASCORBIC ACID, BLOOD ASCORBIC ACID
AND INTRADERMAL TEST TIME OF GUINEA-PIGS.

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In our previous publications (1-2) attempts were made to find how far
the intradermal test could serve as an index for the assessment of the
vitamin C-nutrition of the body. A correlation between the intradermal test
and the blood ascorbic acid level has, however, not been obtained by some
workers (3-6) and they have concluded that the intradermal test is not useful
for the assessment of vitamin C- status of the body, assuming that the blood
ascorbic acid level is the best criterion for the purpose. Drigalski (7) however
observed that serum ascorbic acid level varied by more than 100% in the same
individual on a constant diet and different individuals on the same diet
showed greatly different serum ascorbic acid levels. In minor infections the
serum ascorbic acid level was not much influenced and after injections of
ascorbic acid, there was a great variation in the serum ascorbic acid level in
different individuals. Blood ascorbic acid level, therefore, may not be taken
as a criterion for the assessment of vitamin C-status of the body.

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In the present investigation attempts have been made to explore the relation, if any, between the urinary ascorbic acid, blood ascorbic acid and the intradermal test time under controlled conditions.

EXPERIMENTAL

A group of 17 adult male guinea-pigs of weights varying from 400 g. to 450 g. were fed on an ordinary diet consisting of green grass and germinated gram for a period of three weeks. They were then placed in metabolism cages and their urine collected for 24 hours over 2 cc. of 50% sulphuric acid. Another batch of guinea-pigs, weighing from 200 to 250 g., were placed on a scorbutic diet for a period of three weeks. They were then placed in metabolism cages and their urine was collected as before. The urinary and blood ascorbic acid levels and the intradermal test time with both groups of guinea-pigs were determined. Urinary ascorbic acid was determined by the method of Banerjee *et al* (8). Blood ascorbic acid was determined by the method of Farmer and Abt (9) and the intradermal test time was determined by the method described before (1). The results are given in Tables I and II.

TABLE I

Showing the relation between the urinary ascorbic acid, blood ascorbic acid and the intradermal test time in guinea-pigs on normal diet.

No. of animal	24 Hours' urinary excretion of true free ascorbic acid	Plasma ascorbic acid per 100 cc.	Decolorisation time in the intradermal test.	
1	0.28 mg.	0.69 mg.	2 min.	10 sec.
2	0.38	0.51	1	50
3	0.36	1.74	3	15
4	0.28	1.94	2	30
5	0.36	1.08	4	30
6	0.30	1.19	4	45
7	0.33	0.92	2	45
8	0.34	0.68	3	0
9	0.47	1.49	2	15
10	0.40	1.22	3	15
11	0.33	1.03	1	30
12	0.36	0.94	1	25
13	0.40	0.84	1	30
14	0.58	0.77	5	30
15	0.25	1.06	2	45
16	0.25	0.97	4	15
17	0.61	1.66	4	30

TABLE II

Showing the relation between the urinary ascorbic acid, blood ascorbic acid and the intradermal test time in guinea-pigs on scorbutic diet.

No. of animal	24 Hours' urinary excretion of true free ascorbic acid	Plasma ascorbic acid per 100 cc.	Decolorisation time in the intradermal test.	
1	0.12 mg.	0.38 mg.	15 min.	0 secs.
2	0.02	0.43	12	30
3	0.02	0.42	11	30
4	0.02	0.40	6	0
5	0	0.14	9	0
6	0.16	0.45	7	0
7	0.16	0.67	9	30
8	0.23	0.74	5	0
9	0.03	0.68	10	0
10	1.42	0.78	12	0
11	0.02	1.03	6	0
12	0.01	0.55	7	0
13	0.11	0.58	10	30
14	0.08	0.58	6	30
15	0.25	0.52	6	30
16	0.03	0.51	8	0

DISCUSSION

From the results obtained it is observed that there is no correlation between the blood ascorbic acid level, the twenty-four hours' urinary excretion of ascorbic acid and the intradermal test time either in normal or in scorbutic guinea-pigs. It seems that the urinary excretion of ascorbic acid and the blood ascorbic acid level may depend on various factors and there is no basis in fact for the assumption that the blood ascorbic acid level would be a safe criterion for assessing the ascorbic acid status of the body.

SUMMARY

The urinary excretion of ascorbic acid, blood ascorbic acid and the decolorisation time in the intradermal test have been determined both in a group of seventeen guinea-pigs on normal diet and in another group of sixteen guinea-pigs on scorbutic diet. No correlation between the urinary ascorbic acid and blood ascorbic acid and between urinary or blood ascorbic acid and the intradermal test time has been obtained in either group of guinea-pigs.

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THE INTRADERMAL TEST AS AN INDEX OF VITAMIN C-NUTRITION. PART IV. THE RELATION BETWEEN THE INTRADERMAL TEST TIME AND THE ASCOBIC ACID CONTENT OF THE LIVER OF GUINEA-PIGS.

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It has been observed previously (1) that a minimum time of 1 min. 20-30 secs. (required for the decolorisation of 2:6-dichlorophenolindophenol) ·in the intradermal vitamin C test is obtained both in guinea-pigs and in human beings after administration of massive doses of ascorbic acid. When this minimum time in the skin test is reached we consider that the skin is saturated. It was of interest, however, to investigate whether other organs of the body are also saturated when the skin is saturated, in other words, whether saturation of the skin is an index of saturation of the whole body. In the present work only the liver tissue of guinea-pigs was investigated, as we required a fairly large amount of material and we desired to carry out a thorough investigation of the free and dehydro-ascorbic acid and combined ascorbic acid of liver and of their relation to the intradermal test time.

EXPERIMENTAL

Three groups of guinea-pigs, consisting of 12 in each group, of weights varying between 450 and 550 g. were taken. The first group was kept on a scorbutic diet for a period of three weeks after which the animals were killed. Livers of three animals were minced in a glass mortar. Three 5 g. portions of the minced liver were taken and ground separately with 5 g. of sand and water. Into one sample hydrogen sulphide was passed for 5 minutes in cold condition, then for 15 minutes in hot condition and again for 5 minutes in the cold condition. Into the second sample hydrogen sulphide was passed for 25 minutes in the cold condition. These mixtures were then extracted with trichloracetic acid and centrifuged. The precipitate was washed twice with distilled water and centrifuged. The combined extract was made up to the same volume in each case and after removal of hydrogen sulphide by an

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inert gas, titrations were carried out as usual with 2:6-dichlorophenol-indophenol. In order to specifically estimate the ascorbic acid by indophenol titration, aliquots of the extracts were treated with ascorbic acid oxidase and the values of free, dehydro- and combined forms of ascorbic acid were determined as previously described (2). The free ascorbic acid in the third portion of minced liver was extracted in the usual way with trichloracetic acid and without using hydrogen sulphide. The intradermal test time was determined on the day on which the animals were killed (3).

The second group of guinea-pigs were fed with a normal diet consisting of green grass and germinated gram for three weeks. The intradermal test time was determined on the 22nd day and then the animals were killed and the ascorbic acid content of liver estimated.

The third group of guinea-pigs fed with normal diet were given a daily injection of 100 mg. ascorbic acid for 16 days and killed on the 17th day. The ascorbic acid content of the liver and the intradermal test time on the day of death were determined as before.

The animals of all the above groups were placed in batches of three in metabolism cages, twenty-four hours prior to killing the animals, and their pooled urine was collected over 2 cc. of 50% sulphuric acid. Urinary excretion of free, dehydro- and combined ascorbic acid was determined in all the cases by methods described before (2).

Results obtained with these three groups of guinea-pigs are given in Tables I-III.

TABLE I

Guinea-pigs on scorbutic diet.

No. of animal	24 Hours' urinary excretion of ascorbic acid in mg. in a batch of three guinea-pigs			Decolorisation time in the intradermal test		Mg. of ascorbic acid in 100 g. of liver.			
	Free	Dehydro	Combined			Free	Dehydro	Free and Dehydro	Combined
1.				5 min.	0 sec.				
2.	0.14	0.05	0.21	5	0	2.66	0	2.66	0
3.				7	0				
4.				4	30				
5.	0.21	0.11	0.31	5	0	3.13	0	3.13	0
6.				5	0				
7.				5	30				
8.	0.13	0.15	0.16	9	15	3.13	0	3.13	0
9.				7	20				
10.				4	30				
11.	0.17	0.14	0.38	died		3.13	0	3.13	0
12.				died					

TABLE II
Guinea-pigs on scorbutic diet.

No. of animal	24 Hours' urinary excretion of ascorbic acid in mg. in a batch of three guinea-pigs			Decolorisation time in the intradermal test		Mg. of ascorbic acid in 100 g. of liver.			
	Free	Dehydro	Combined			Free	Dehydro	Free and Dehydro	Combined
1.				3 min. 30 sec.					
2.	1.40	0.18	1.17	3	0	15.32	7.18	22.50	0
3.				5	0				
4.				4	0				
5.	1.69	0	0.72	3	0	13.15	14.04	27.19	0
6.	—			6	30				
7.				3	0				
8.	1.57	0.12	0.45	2	30	6.38	0	6.38	0
9.				3	0				-
10.				3	15				
11.	2.31	0.21	0.75	2	30	5.96	9.93	15.89	0
12.				2	30				

TABLE III
Guinea-pigs on normal diet receiving injections of 100 mg. ascorbic acid.

No. of animal	24 Hours' urinary excretion of ascorbic acid in mg. in a batch of three guinea-pigs			Decolorisation time in the intradermal test		Mg. of ascorbic acid in 100 g. of liver.			
	Free	Dehydro	Combined			Free	Dehydro	Free and Dehydro	Combined
1.	143.30	0	0	1 min. 30 sec.		21.00	10.27	31.27	11.90
2.				1	30				
3.				1	30				
4.	62.29	10.10	0	1	30	14.52	5.48	20.00	2.78
5.				1	30				
6.				1	30				
7.	92.64	1.65	0	1	30	17.73	2.51	20.24	5.06
8.				1	30				
9.				1	30				
10.	103.65	0	0	1	30	17.13	1.09	18.22	2.17
11.				1	30				
12.				1	30				

DISCUSSION

It is not clear whether the figures for dehydroascorbic acid of the liver given in tables I-III are significant, as it is conceivable that it is not present as such in the liver but is formed in the course of grinding the tissue (4). For our present purpose the sum of the free ascorbic acid and dehydroascorbic acid values of liver may be taken as the free ascorbic acid. The ascorbic acid content of the liver is greatly diminished in guinea-pigs on a scorbutic diet, the value being 2.66 -3.13 mg. per 100 g. of liver. The urinary excretion of free ascorbic acid per animal per day is 0.05 mg. The intradermal

test time is highest in this group of guinea-pigs, 4 min. 30 secs. to 9 min. 15 secs. In the second group of guinea-pigs on normal diet free ascorbic acid content of liver varies from 6.30 to 27.19 mg. The urinary excretion of ascorbic acid in this group is 0.58 mg. The intradermal test time varied in this group between 2 min. 30 secs. and 5 min. In the third group getting a supplement of ascorbic acid there is less variation in the ascorbic acid content of liver of the different animals, the values ranging from 18.22 to 31.27 mg. being of a very high order. The daily urinary excretion of ascobic acid is the highest in this group (33.82 mg). The intradermal test time is the minimum, 1 min. 30 secs., in all the animals. It will thus be observed from a comparison of the three groups of animals that the liver ascorbic acid and urinary ascorbic acid values steadily rose and the intradermal test time steadily declined with increasing doses of ascorbic acid. In the third group, the diminished variation in the liver ascorbic acid from animal to animal indicates that perhaps the liver ascorbic acid value was reaching its maximum. At this stage the intradermal test time reached its minimum value. This would seem to indicate that when the saturation stage is indicated by the skin test, the liver also tends to be saturated with vitamin C. If that is so this would be additional evidence to indicate that the skin test would afford a fair guide for the vitamin C-saturation of the body.

SUMMARY

The free, dehydro- and combined ascorbic acid content of the liver, the urinary excretion of these different forms of ascorbic acid and the intradermal test have been determined in three groups of guinea-pigs kept respectively on scorbutic diet, normal diet and normal diet with a supplement of ascorbic acid by injection. The intradermal test time was highest in the group receiving scorbutic diet and lowest in the group receiving injections of vitamin C.

The free ascorbic acid content of the liver and urine varied inversely in a rough manner as the decolorisation time in the intradermal test. The livers of the animals dosed with excess of vitamin C appear to reach a maximum value in ascorbic acid and at the same time the minimum value is reached in the skin test. These results appear to indicate that the intradermal test affords a fair index for the vitamin C saturation of the body.

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THE INTRADERMAL TEST AS AN INDEX OF VITAMIN C-NUTRITION. PART V. THE RELATION OF THE DECOLORISATION TIME IN THE INTRADERMAL VITAMIN C-TEST AND THE DISAPPEARANCE OF COMBINED ASCORBIC ACID IN THE URINE OF GUINEA-PIGS.

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In previous communications (1-4) attempts have been made to show how far the intradermal test is suited for the assessment of vitamin C-nutrition of the body. It has been shown by us (5) that combined ascorbic acid disappears from the urine of normal and tubercular subjects and of guinea-pigs after the prolonged administration of ascorbic acid in large doses. It has been suggested that in the absence of ascorbic acid the normal metabolism of the foodstuffs and of tissue constituents may be disturbed and some intermediary products may accumulate, which may be excreted in combination with ascorbic acid in urine. When large doses of ascorbic acid are fed, the metabolism is complete and the intermediary products do not accumulate and therefore the excretion of ascorbic acid in combination with these products would tend to diminish. This may happen both in normal and in infected conditions, and the quicker disappearance of combined ascorbic acid from the urine of normal persons by feeding ascorbic acid may be related to the possibly greater speed in the action of vitamin C in normal individuals. If this assumption be correct the disappearance of combined ascorbic acid from urine would also suggest a means of determining the vitamin C-status of the body. It was of interest to know whether the disappearance of combined ascorbic acid from urine would denote the saturation of the body. In the present investigation attempts have been made to study the relation of the decolorisation time in the intradermal vitamin C-test as an index of saturation and the disappearance of combined ascorbic acid in the urine of guinea-pigs.

EXPERIMENTAL

Fourteen healthy male guinea-pigs of weights varying from 400 to 450 g. were taken. Prior to the investigation the animals were receiving an ordinary diet consisting of green grass and germinating gram for a period

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of three weeks.   The animals were kept in metabolism cages in batches of two and their twenty-four hours' urine was collected over 2 cc. of 50% sulphuric acid.   After collection of 24 hours' urine all the animals were injected with 100 mg. of ascorbic acid and 24 hours' output of urine was again collected.   Free, dehydro- and combined ascorbic acid excretion in the urine was determined.   It was found that combined ascorbic acid disappeared from the urine after the animals received a single injection of ascorbic acid.   The intradermal test time was determined when the combined ascorbic acid disappeared from the urine.   The animals were killed on the day of disappearance of combined ascorbic acid and the free ascorbic acid content of the liver was estimated.   The results are given in Table I.

TABLE I

Showing the relation among the intradermal test time, the disappearance of combined ascorbic acid in the urine and the ascorbic acid content of the liver of guinea-pigs injected with 100 mg. ascorbic acid.

Animal No.	24 Hours' urinary excretion of ascorbic acid in mg.						Decolorisation time in the intradermal test done on the day of disappearance of combined ascorbic acid in urine.		Ascorbic acid content of liver in mg. per 100 g. of liver of guineapigs killed on the day of the disappearance of combined ascorbic acid in urine.
	Before injection of ascorbic acid			After injection of ascorbic acid					
	Free	Dehydro	Combined	Free	Dehydro	Combined			
1. 2.	0.76	0	0.16	29.17	0	0	3 min. 1	0 sec. 55	17.2 11.6
3. 4.	0.88	0.12	0.75	40.0	0	0	2 2	20 10	23.8 21.8
5. 6.	1.00	0	0.21	47.62	0	0	2 3	45 0	12.6 13.6
7. 8.	1.21	0	0.25	43.47	0	0	2 2	45 30	15.2 14.2
9. 10.	1.31	0	0.89	33.33	0	0	3 3	0 0	11.2 10.0
11. 12.	1.21	0	0.25	45.45	0	0	2 3	45 15	25.0 24.4
13. 14.	0.89	0	0.89	50.00	0	0	2 2	30 15	16.6 16.4

DISCUSSION

From the results it is observed that comined ascorbic acid which was present in the urine of guinea-pigs receiving a normal diet for a period of three weeks disappeared from the urine one day after the injection of 100 mg. ascorbic acid. There was also a peak in the urinary excretion of free ascorbic acid along with the disappearance of combined ascorbic acid. The decolorisation time in the intradermal test on the day of disappearance of ascorbic acid, however, varied from 1 min. 55 secs. to 3 min. 15 secs. In no case was the decolorisation time reduced to 1 min. 30 secs. which, as has been shown (2), denotes saturation of the body if the intradermal test time is taken as the index for the assessment of vitamin C-nutrition of the body. The disappearance of combined ascorbic acid from the urine would not, therefore, seem to indicate body saturation with vitamin C. The ascorbic acid content of the liver likewise varied from 11.6 to 25 mg. per 100 g. of liver, which is also lower than the ascorbic acid content (18.2—31.3 mg.) of the liver when the minimum decolorisation time is reached in the skin test, as we have observed before (4). Under these circumstances it would seem that the disappearance of combined ascorbic acid would not denote the saturation of the body but it would probably indicate a good degree of vitamin C-nutrition. It is conceivable that this stage corresponds to a state of optimum nutrition, without the body being necessarily saturated. But whether this is actually so requires to be determined. These results would seem to make it desirable to drop the words like 'saturation tests', which have been used in the literature in this connection, for it is clear that in the conditions studied by these tests strict saturation of the body with vitamin C never occurred.

SUMMARY

The decolorisation time in the intradermal test has been studied in a group of fourteen male guinea-pigs, each receiving an injection of 100 mg. ascorbic acid for one single day. The combined ascorbic acid disappeared from the urine one day after injection of ascorbic acid and there was also a peak in the urinary excretion of ascorbic acid. The intradermal test time varied from 1 min. 55 secs. to 3 min. 15 secs. The animals were thus not saturated with vitamin C as the minimum time of 1 min. 20 to 30 secs. in the decolorisation time was not obtained. The disappearance of combined ascorbic acid does not, therefore, mean that the body is saturated with vitamin C. The vitamin C content of the liver of the above animals killed on the day of disappearance of combined ascorbic acid varied from 11.6 to 20 mg. per 100 g. of liver, which is also less than that obtainable with massive doses of vitamin C. This condition probably represents a good state of

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vitamin C-nutrition without the body being necessarily saturated with vitamin C. It seems desirable to make a clear distinction between optimum nutrition and saturation.

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Obituary

Dr. P. de Braganca-Cunha

We grieve to announce the premature death of Dr. Braganca-Cunha in the early hours of 12th November, 1942.

Dr. Braganca-Cunha was born on the 4th October, 1894 in Goa. He received his medical education in Calcutta and started practice here twentyfive years ago. He rose to eminence as a practising physician of this city and brought a particularly scientific mind to bear on his clinical practice. The Indian Institute for Medical Research had in him an abiding friend from its inception. He was not only a member of the Governing Body of the Institute but was an intimate associate and inspirer in its day-to-day work. His contact with us was both scientific and personal and his passing away creates a sense of void, which time would hardly heal. Yet we trust our deep-felt homage would really be worthy of him if we can steadily further the bounds of knowledge, which he had so much at heart.

Those who had the privilege of coming into personal contact with Dr. Braganca-Cunha will not easily forget him. Generous to a fault, of even temper, with kindness written on his countenance, of extra-ordinary culture and charm, he was a man,

whose memory it is an inspiration to cherish. He believed in truth and love and in the socialist order of life. Though born a Catholic, he believed in no denominational religion and desired a civil burial. The civil war of humanity that is now going on tore his heart and he longed for the day when socialism would arise on the ashes of international greed. Nearest to his heart was the cause of the freedom of the people of India and nobody who came into intimate touch with him failed to receive the spark of his consuming patriotism.

The Indian Institute for Medical Research will strive its utmost to see that his name is permanently associated with it and that his message is carried forward in humble devotion and sincerity.

ACTION ON THE HÆMOPOIETIC SYSTEM OF MONKEYS OF
DRUGS OF THE SULPHONAMIDE GROUP ADMINISTERED
IN THERAPEUTIC DOSES

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Since the discovery of the bactericidal properties of the 'sulphonamide group of drugs, these have been widely employed in all conceivable diseases in clinical therapeutics and it is generally conceded that these remedies have provided the medical profession with a very useful means of combating bacterial diseases. A disadvantage which is found frequently associated with the clinical use of these drugs is that they are likely to bring about a state of anæmia in the patients.

Clinicians have reported observations on hospital patients which leaves little room for doubt that the sulphonamides are responsible for blood destruction, at least in high doses. Wood (1) has reported 21 cases of acute anæmia seen at the John Hopkins Hospital in patients treated with sulphanilamide, the largest dose given being 0.2 g. per kg. of body-weight in 24 hours. Jennings and Sander (2) have reported the onset of slow anæmia following a course of the drug, the maximum anæmia occurring between two and four weeks after the start of the treatment. Harvey and Janeway (3) reported cases from John Hopkins Hospital which developed acute hæmolytic anæmia during the administration of sulphanilamide. Large doses resulted in destruction of red blood cells and of hæmoglobin, appearance of nucleated red cells

and urobilinuria. The condition however promptly improved and symptoms disappeared after transfusions of citrated blood. Development of acute hæmolytic anæmia in patients during treatment with sulphanilamide has also been described by Kohn (4). Remington (5) has observed that patients and animals receiving the drug showed an increased excretion of porphyrin in the urine. The little experimental work that is traceable on the subject is as follows ; Hægeman (6) reported the finding of splenic hæmosiderosis in mice to which sulphanilamide has been administered and he attributed the deposits of hæmosiderin to destruction of erythrocytes in the peripheral blood. Finklestone et al (7) found little or no change in the erythrocyte or reticulocyte levels of rabbits to which a daily dose of 1.2 g. of sulphanilamide was given for six weeks, but abnormal and nucleated erythrocytes in the peripheral blood appeared. Wein and Quart (8) found lower erythrocytes and hæmoglobin levels in 3 rats receiving 1.5 g. of sulphanilamide per kg. of body-weight. No depression was, however, noticed in rats which received 0.5 g. of the drug. Davis et al (9) reported reduction in the number of red blood cells and in the amount of hæmoglobin together with a slight leucocytosis in rats which received daily subcutaneous doses of su'phanilamide. The doses were 0.5, 1.0, 1.5 and 2 g. per kg. of body-weight and administration was continued over prolonged periods. Machetta and Higgins (10, 11) reported that the prolonged administration of sulphanilamide to rats produced an anæmia characterised by reticulocytosis, increased diameter of the red cells and hyperplasia of bone marrow. The doses used were from 0.25 g. to 2 g. per kg. of body-weight. The doses administered by the above mentioned workers have been much higher than what is usually given therapeutically. The results indicate that in doses beyond the usual therapeutic limits, sulphonamides undoubtedly bring about anæmia characterised by extensive cell destruction and other evidence of injury to the bone marrow.

In view of the fact that little or no work has been reported on the response of the blood-forming organs, produced by therapeutic doses of the sulphonamides, as opposed to toxic doses, it was thought desirable to take up this study on monkeys. The reasons for selecting this animal are not far to seek. Monkey is an ideal experimental animal in as much as it is biologically and physiologically very close to the human being. Hæmatologically too, it was found (loc. cit.) that this animal is very close to human being. The clinical bearing of such a study is obvious and it is expected that the results would be applicable to human beings in toto. It would further provide much needed informations to clinicians prescribing such drugs in low, run down and already anæmic patients. Therefore, while proposing to study the action of these drugs on the hæmopoietic system in normal monkeys, it was found essential to ascertain a normal standard of the different elements of blood of normal healthy monkeys. Accordingly the following investigations· were carried out.

PART A

Hæmatological studies in normal monkeys
(Silenus Rhesus) in Calcutta

EXPERIMENTAL METHODS

Animals used.—Healthy monkeys (*Silenus Rhesus*) weighing 3.2 kg. to 5.2 kg. each were placed in separate cages under identical conditions on a standard diet (consisting of 4 oz. gram, 4 oz. green vegetables, and 4 oz. bananas daily). Water was allowed *ad libitum*. A week was allowed for the animals to get accustomed to the laboratory conditions and to the diet before experiments were started, then complete blood examination was done weekly. Four such examinations were carried out with each animal and its weekly weights recorded.

Technique.—All examinations were made on venous blood partly because it is more convenient and accurate to work on venous blood and partly because all normal hæmatological standards in man have been done on venous blood (*cf.* Wintrobe, 12 ; Jones, Vaughan and Goodard, 13 ; Napier and Das-Gupta, 14 ; Sockey *et al*, 15). Blood samples were collected between 11 A.M. and 12 noon from fasting animal by venupuncture of the leg vein with a dry syringe ; a small drop was taken on to a slide for making films for differential white cell count, and 3 ccs. of blood were then drawn into an oxalated flask containing a mixture of potassium and ammonium oxalates. It was found that the blood of monkeys coagulated very quickly in the syringe rendering withdrawal of blood very difficult, hence coagulation time of blood was recorded in each case. Standard methods were used for determining the various constituents of the blood.

(*i*)   *Coagulation time.*—The capillary tube method was followed, the temperature being 37°.

(*ii*) *Hæmoglobin Estimation.*—This was done by Hellige's method in the Hellige normal Hæmometer.
  Hellige 100% reading 13.75 g. of Hgb. per 100 cc. of blood.

(*iii*) *Red and White Cells Count.*—These were done on Neubauer ruling chamber. Counting and calculations were done by standard methods. The average of two counts was taken.

(*iv*) *Reticulocyte* counts were made by mixing equal parts of blood and brilliant cresyl blue solution in normal saline. The number of reticulocytes to 1000 red cells was counted.

(v) *Blood films* were stained by Leishman's stain and differential white cell count was done, counting 200 cells each time. Neutrophil leucocyte lobes were counted by Arneth method modified after Cooke and Ponder and weighted mean was calculated.

(vi) *Cell volume* was estimated by Wintrobe's technique.

Mean Cell Volume (M.C.V.), *Mean Corpuscular Hæmoglobin* (M.C.H.) and *Mean Corpuscular Hæmoglobin Concentration* (M.C.H.C.) were calculated by standard methods.

Table I gives the summary of hæmatological examination of 10 normal monkeys.

Table II summarises the hæmatological findings of 50 male Indians of Calcutta (Napier and Das-Gupta) and the mean findings of both tables (I and II) have been subjected to a close statistical study in Table III.

TABLE I

Summarising hæmatological findings of '40 complete blood examinations on normal monkeys (showing findings in whole series.)

	Maximum	Minimum	Mean	Standard error
Red Cells per cmm. in millions	6.33	4.53	5.30	±0.068
Hgb. (g.) per 100 cc. of blood	16.50	11.69	13.28	±1.70
Per cent of 13.75	120	85	96.62	±1.30
Reticulocytes per cent of total r.b.cs.	3.5	0.8	1.46	±0.11
Volume of packed cells: percentage of whole body	53.5	36.0	41.96	±0.62
Mean corpuscular volume (M.C.V.) in (cubic microns) cu. μ	92.4	61.3	78.96	±1.17
Mean corpuscular hæmoglobin in micro-micro g ($\gamma\gamma$)	29.3	20.2	25.08	±0.32
Mean corpuscular Hgb. concentration%	37.1	27.2	31.82	±0.35
Total white blood cells per cmm.	19.900	5.200	13.689	±542.9
Neutrophils per cent of white cells	80	21	41.73	±1.87
Lymphocytes ,, ,, ,,	72	15	52.4	±1.91
Large mononuclear- cells ,, ,,	9	1	3.9	±0.29
Eosinophils ,, ,,	7	1	2.2	±0.21
Arneth count	2.06	1.32	1.66	±0.07
Coagulation time	60 sec.	40	42	±1.60
Van den bergh reaction	-ve	-ve		

TABLE II

Summarising Hæmatological findings in 50 adult Male Indians Working in Calcutta. (By Napier and Das Gupta, 14, 16.)

	Maximum	Minimum	Mean	Standard deviation
Red cells per cmm. in millions	6.7	4.1	5.3	± 0.633
HgB. (g.)	17.19	11.00	14.77	± 1.36
Percent of 13.75	125	80	107.44	± 9.86
Reticulocytes % of total red cells	1.4	0.2	0.67	± 0.37
Platelets per cmm.	1020000	56000	369000	± 248000
White blood cells per cmm.	9600	4080	6542	± 1214
Eosinophils % of total white cells	18	none in 250 cells	6.91	± 5.19
Large mononuclear cells per cent of Total white cells	23	2	7.00	± 3.91
Volume of packed cells	55.60	44.10	50530	± 3.02
Mean corpuscular volume in cubic microns	105.30	72.70	90.49	± 7.90
Mean corpuscular HgB. in micro-micro g.	32.60	21.60	28.53	± 2.31
Mean corpuscular HgB. Concentration in per cent	34.10	28.60	31.07	± 1.20

TABLE III

The following table summarises the results of the significance tests carried out.

	Adult male Indian mean	Monkey's mean	Value of t	Significance
Red cells per cmm.	5320000	5305000	0.12	Not significant
HgB. (g.) per 100 ccs. of blood	14.77	13.28	5.73	Significant at 1%
Per cent of 13.75	107.44	96.62	5.55	,,
Reticulocyte per cent of total red cells	0.67	1.46	7.18	,,
White blood cells per cmm.	6542	13689	43.34	,,
Eosinophils per cent of total white cells	6.91	2.2	5.61	,,
Large mononuclear cells per cent of W.B.Cs.	7.00	3.9	4.70	,,
Volume of packed cells percentage of whole blood	50.53	41.96	10.27	,,
Mean corpuscular volume (M.C.V.) in cubic microns	28.53	25.08	6.69	,,
Mean corpuscular HgB. % Conc.	31.07	31.82	1.74	Not significant

DISCUSSION

A normal hæmatological standard of the different elements of monkey's blood was thus ascertained from 40 complete blood examinations as in Table I. A comparative study was done of this normal standard with that of the normal hæmatological findings in healthy adult males in Calcutta by Napier and Das Gupta (14, 16) as in Table II, the mean and the value of t were ascertained as in Table III.

The value of t give the exact probability for the given difference between the means or for higher differences resulting from random samples, when pairs of samples of the given sizes are taken. If the value of t is 1.99 the difference between the means is significant at the 5 per cent level and if it is 2.63 the difference is significant even at the 1 per cent level. For smaller values of t than 1.99 it is safe to assume that the difference observed in the means is due to chance.

It is interesting to note that on comparison it is found that the mean values of red blood cells per cmm. and mean corpuscular hæmoglobin concentration in the monkey's blood correspond closely with those of human beings, but the monkey and human bloods differ significantly in respect of other elements of blood, the figure for monkeys being higher than that for human beings in respect of reticulocyte, percentage of total red cells and white blood cells.

PART B
The effect of sulphonamide group of drugs on blood.

The effects on the hæmopoietic system of therapeutic doses of (1) Prontosil soluble, (2) Prontosil album (sulphanilamide) and (3) M. & B. 693 (sulphapyridine) were studied on the monkeys previously used for estimating normal hæmatological standards.

Animals and their treatments.—The monkeys as before were kept under controlled conditions with regard to diet etc., in the laboratory. All animals were fasted overnight and the drugs were administered through a stomach tube in the morning, in empty stomach to ensure maximum absorption. The dose in each animal was repeated daily. The animals were divided into five groups and treated in the following manner.

(i) In Group I, three monkeys (No. 1, 3 and 4) were given 'Prontosil soluble' (Bayer) as 5% solution by intramuscular injections into the gluteal muscles daily. Animals No. 1 and 3 each received a daily dose of 0.06 g. per kilo body-weight ; in the former the drug was continued for 20 days while in the latter it was given only for 15 days. Animal No. 4 received 0.09 g. (per kilo body-weight) which was continued for only one week.

(ii) The second group of animals (No. 5, 7 and 8) were given finely powdered 'Prontosil album' in water suspension for 20 days.

Animals No. 5 and 7 received a daily dose of 0.06 g., while No. 8 received a dose of 0.09 g. per kilo body-weight.

(iii) In the third group, animals (No. 10 and 12) were given per os, a daily dose 0.06 g. (per kilo body-weight) of M. & B. 693, powdered and suspended in a fair amount of water. It was however noticed, as pointed out by Marshall et al (17), that M. & B. 693 was only partially soluble in water. It was therefore administered to the next group of animals in a soluble form.

(iv) The fourth group of animals (No. 11 and 13) were therefore given a daily dose of 0.06 g. of the drug in acid solution. For this purpose it was dissolved in dilute hydrochloric acid (by warming on the water-bath, if necessary) and the pH of the solution was adjusted approximately to that of the gastric juice by the addition of a saturated solution of sodium bicarbonate.

(v) The fifth group consisted of animals No. 2 and 6 which were kept as controls.

Blood examination was done daily in the animals receiving the drug by injection and twice weekly in animals receiving it by mouth. In the case of control animals, weekly blood examinations were done. The same procedure was also followed during the recovery period following the withdrawal of the drug.

The technique of blood collection and examination is the same as described in Part I of this paper. Blood concentration studies were also frequently carried out after the administrations of the drugs, 2 cc. samples of blood being withdrawn at fixed intervals from one of the femoral veins by means of an accurately calibrated syringe. The method adopted for analysis was that of Marshall, Emerson and Cutting (18) as modified by Doble and Greiger (19).

EXPERIMENTAL RESULTS

General observations.—It is difficult to interpret the symptoms and clinical signs noticed in monkeys during the drug therapy, but some definite signs are worth mentioning. The animals (in Group I) showed marked restlessness immediately after the injection, which continued for $\frac{1}{2}$ to 1 hour and was followed by drowsiness and apathy for several hours.

Diarrhœa was noticed in two animals of Group II (No. 5 and 8). In animal No. 5, the diarrhœa was fairly acute and this probably accounted for the pronounced fall in weight in that animal (initial weight, 8.5 lbs. and terminal weight, 5.5 lbs.). Weight-loss of 1-2 lbs. was also noticed in other animals, whereas the controls, No. 2 and 6, gained in weight. In the beginning loss in weight was somewhat slower in comparison with the production of anæmia. Recovery from anæmia and from loss of weight occurred rapidly after withdrawal of the drugs and the animals reached their normal levels in two to four weeks.

TABLE IV.

Experimental Results.

Animal No. & Sex.	Daily dose of drug administered.	Duration of Drug Administration in days.	Blood conc. of drug max. mgm. %	Total amt. of drug given in g.	Loss of wt. in lbs.	Clinical observation.	Onset of anaemia.	R.b.cs. per cmm. in mill. max. decrease.	Hgb. in g. max. decrease.	Reticulocytes % Max. increase.	M.C.V. increase.	Total leucocytes per cmm. Max. decrease.	Arneth count increase.	Coagulation time in sec. increase.
GROUP I.														
1-F.	Prontosil Soluble by injection 0.06 g. kg. daily.	20	—	5.0	1.0	—	4th day	1.385	2.65	7.0	11.1	8575	0.51	25
3-F.	-do-	15	—	3.75	1.0	—	2nd day	1.780	2.78	5.0	30.15	5700	0.34	60
4-F.	0.09 g. kg. daily	7	1st day 31.5	2.63	2.0	—	,,	1.480	4.06	13.5	20.4	3263	0.28	25
GROUP II.														
5-M.	Prontosil Album 0.06 g. kg. daily stomach tube.	20	—	4.8	3.0	Diarrhœa ++	4th day	0.920	2.72	3.4	12.66	7100	0.06	5 sec. decrease
7-F.	Prontosil Album per Os. 0.06 g. kg. daily.	20	1st day =6.83 8th day =7.5	5.12	1.0	—	2nd day	1.915	2.08	2.7	57.43	—	0.19	20 sec. increase
8-M.	Prontosil Album per Os. 0.09 g. kg. daily.	20	1st day =6.45 8th day =8.76	6.0	2.0	Diarrhœa ++	4th day	0.562	2.37	1.0	10.8	7150	0.24	35

TABLE IV (Contd.)

Animal No. & Sex.	Daily dose of drug administered.	Duration of Drug Administration in days.	Blood conc. of drug max. mgm. %	Total amt. of drug given in g.	Loss of wt. in lbs.	Clinical observation.	Onset of anæmia.	R.b.cs. per cmm. in mill. max. decrease.	Hgb. in g. max. decrease.	Reticulocytes % Max. increase.	M.C.V. increase.	Total leucocytes per cmm. Max. decrease.	Arneth count increase.	Coagulation time in sec. increase.
GROUP III.														
10-M.	M & B 693 per. Os. 0.06 g. kg. in water daily.	20	1st day =2.67 4th day =3.04	4.84	2.5	—	4th day	2.050	4.05	4.15	34.5	4035	0.40	45
12-F.	M & B 693 per Os. 0.06 g. kg. in water daily.	7	1st day =2.66 7th day =3.12	1.8	1.5	—	4th day	0.996	1.53	2.7	3.2	5100	0.20	10
GROUP IV.														
11-M.	M & B 693 per Os. 0.06 g. kg. in Hcl. daily.	20	1st day =5.0	5.14	1.5	—	4th day	1.545	3.46	4.31	8.7	8140	0.30	58
13-F.	M & B 693 per Os. 0.06 g. kg. in Hcl. daily.	7	15th day =9.28	2.3	0.5	—	3rd day	1.157	3.58	1.8	2.9	4550	0.30	—
GROUP V.														
2-M.	Control.				*1.0		,,	†0.605	†2.37	—	—	—	—	—
6-M.	Control.				*1.5		,,	†0.515	†2.27	—	—	—	—	—

*gain in wt.   †increase.

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EXPERIMENTAL DATA

A summary of the experimental findings is given in Table IV. It will be seen that the earliest signs of anæmia, as manifested by a fall of hæmoglobin and total red blood cells, appeared between 24 and 72 hours after the onset of treatment by both Prontosil and M and B. 693. The maximum anæmia in all cases occurred within 7 to 10 days, after which a balance appears to be set up, which is not disturbed even if the drug is continued for 20 days, the blood picture remaining more or less at the same level. There is often a slight tendency to improvement in some cases. There is a progressive decrease of hæmoglobin and total red cells. The M.C.V. and the percentage of circulating reticulocytes are increased. Total leucocytes are decreased in cases where Prontosil (by injection) and sulphapyridine are administered; but they are variable when Prontosil is given orally. Arneth count has a tendency to rise to some extent in all cases. Immature red cells, megaloblasts, erythroblasts' and normoblasts were found in the circulating blood of animals treated with Prontosil by injection and a few normoblasts were found in those treated with the hydrochloride of M and B. 693. Differential leucocyte count shows that during the period of administration of these drugs neutrophil leucocytes increase in number and the lymphocytes decrease to some extent, in nearly all cases. Van den bergh reaction is negative in all cases. The coagulation time is increased in all the animals except in monkey No. 5, where it has slightly decreased. This animal suffered from marked diarrhœa which may account for this difference.

Changes in the different elements of blood in each animal is shown in the accompanying charts (in the appendices) and, in the different group of animals with (i) different preparations of the drug and (ii) their varying dosage are as follows:

(i) *Red blood cells.*—Average maximum decrease in the number' of red cells in millions per cmm. in animals of Group I treated with 0.06 g. of sulphanilamide by intramuscular injection is 1.582 as compared with 1.417 in animals of Group II treated with the same dose of 0 06 g. per kilo body-weight daily by stomach tube. In Group I, treated with 0.09 g. of drug per kilo body-weight daily decrease is 1.480 compared to 0 562 in animal of Group II treated with the same dose orally. Average red cells decrease in animals of Group III was 1.523 as compared to 1.351 decrease in animals of Group IV.

(ii) *Hæmoglobin in g.*—Average maximum decrease in animals of Groups I and II (treated with small doses of the drugs) was 2.72 g. and 2.4 g. respectively. With larger doses the decrease was 4.06 g. in Group I and 2.37 g. in Group II. The average maximum

decrease in animals of Group III was 2.79 g. as compared to 3.61 g. of animals of Group IV.

(iii) *Mean Corpuscular Volume.*—Maximum average increase in animals treated with 0.06 g. per kilo body-weight daily, was 20.58 cubic microns in Group I and 35.03 in Group II. M.C.V. was also increased on M and B. 693 therapy by 18.9 cubic microns in animals of Group III and by 10.8 in animals of Group IV.

(iv) *Total Leucocytes.*—Average maximum decrease with smaller dose was 7.157 per cmm. in animals of Group I and 3.500 in animals of Group II. With the larger dose decrease was 3.263 in Group I and 7.150 in Group II. Average maximum decrease in Group III was 4,567 as compared to 6.345 in Group IV.

(v) *Arneth Count.*—Increase was 0.42 in animals of Group I with the smaller dose, as compared to 0.12 increase in animals of Group II treated with the same dose. With the larger dose increase in animals of Group III was 0.30, which is more or less the same as in animals of Group IV.

(vi) *Coagulation time.*—Increase was 42 seconds in animals of Group I treated with the smaller dose and 7.5 seconds in Group II animals treated with same dose ; 25 seconds increase in Group I animals treated with the larger dose and 35 seconds increase in Group II animals treated with the same dose. Increase was 27.5 seconds in Group III animals and 29 seconds in animals of Group IV.

Results of Biochemical Findings.—The concentrations of the drugs in blood found after the oral administration of sulphanilamide and sulphapyridine and that following the intramuscular injection of 'Prontosil soluble' are as follows :—

The maximum concentration reached in the blood after the first day of administration of sulphanilamide by mouth were 6.45 and 6.83 mg. per cent, maximum reaching about $1\frac{1}{2}$ hours after the administration of the drug as in the following graphs of blood concentration study of animals No. 8 and animal No. 7.

With sulphapyridine in water, the figures are very much lower, being 2.66 and 2.67 mg. per cent and maximum was arrived at 1 to 2 hours later as in the following graphs of blood concentration study of animals No. 10 and 12.

When sulphapyridine was administered in acid solution, the maximum concentrations in both animals were 5.0 mg. per cent or very near to the figures arrived at in case of animals receiving an identical dose of sulphanilamide. The maximum concentration was also reached in $1\frac{1}{2}$ hours as shown in the following graphs of blood concentration study of animals No. 11 and 13.

It was found that when successive administrations of the drugs have been made over a number of days, a small quantity of the drug was still found to persist in the blood even after 24 hours and a fresh dose caused the drug concentration to rise to a level very much higher than that which was recorded on the first day of drug administration.

The blood concentration after injection of 'Prontosil Soluble' in animal No. 4, as in the following graph, demonstrates that the figures obtained are several times higher than in cases where the drug was given orally. Maximum concentration was reached after $\frac{1}{2}$ hour and the drug was completely eliminated from the blood within $2\frac{1}{2}$ hours ; the rates of absorption and elimination were much quicker.

A comparative study of the blood concentrations of sulphanilamide and sulphapyridine has already been published (20).

DISCUSSION

The foregoing pages embody the results of experiments carried out on monkeys with a view to find out the toxic effects of the drugs of the sulphonamide group in *therapeutic* doses on the hæmopoietic system. Many workers, mostly in the United States have done experiments on various animals in this connection, but the doses employed by them were usually toxic, and not therapeutic. Rabbits and mice were mostly used as experimental animals. Monkey has been chosen for the purposes of present investigations for reasons stated before.

It is well known that one of the effects of administration of these drugs on human hæmopoietic system is to produce an arrest of the normal leucopoiesis as it occurs in the bone marrow. This is evidenced by progressive diminution of white blood cells in the peripheral circulation. Innumerable cases are on record where the administration leads to a condition so well known as malignant neutropenia or agranulocytosis. In fact, it has become a practice in most hospitals to always have a total white cell count of the patients before and during the course of the sulphonamide therapy. In my experiments, there is also to be noticed a consistent decrease of white cells in all monkeys.

It is, however, for the effect of these drugs on erythropoiesis as it occurs in the hæmopoietic tissues that the experiments were planned. Very little work has been done on this side of the blood picture, either in India or abroad. Various clinicians have observed the onset of anæmia or at least pallor in cases treated with such drugs, but very little work has been done to confirm this observation. Findings recorded above show the following significant changes in the blood picture of the monkeys, compared with the controls:

(i) Progressive and moderate reduction in the number of red blood cells, and the total hæmoglobin. This decrease seems to occur in most animals from the 2nd to 4th day of the administration of drug. The maximum decrease in the red cells ranges between 1 and 2 million per cmm., and is accompanied by a reduction in the total hæmoglobin of about 1.5 to 3 grams per cent.

(ii) The M.C.V. showed an increase in almost all animals, showing a very wide range, 2.9 to even 50 cubic microns. The anæmia on the whole seemed to be accompanied by increase of cell volume.

(iii) The reticulocyte count also showed an increase in the number, varying from 2 to 15%.

(iv) It was also noticed that the maximum increase in the number of reticulocytes took place almost on the same day as the maximum reduction in the number of red cells was noticed.

(v) Van den bergh's reaction was found throughout the investigations to be negative.

From the above findings there is little doubt left to show that the administration of the sulphonamide group of drugs even in the therapeutic doses is accompanied by a moderate and progressive anæmia. The pathogenesis of anæmia thus produced is far from being clear and well defined. There are two possible ways in which the anæmia may be produced.

(1) The sulphonamides might be responsible for depressing the activity of the normal hæmopoietic tissues. These drugs may bring about a maturation arrest of the red blood cells as they do in the case of the white blood cells.

(2) The anæmia produced may be due to hæmolysis of the red cells brought about by the drug in the circulation.

Wood (1), as also Jennings and Sanders (2), attributed the anæmia occurring in patients during sulphonamide therapy to hæmolysis of red cells. They found a positive Van den bergh reaction in their cases as also urobilin in the urine. Hægeman (6), and Remington and Hemming (5) found deposits of hæmosiderin in liver and spleen on necropsy in experimental animals treated with sulphonamides. On the whole, therefore, the evidence points to a hæmolytic origin of anæmia. In the present series of experiments there are certain points which confirm the same finding, namely, reticulocytosis corresponding with the degree of anæmia. The Van den bergh reaction, however, was negative throughout in all the experimental animals. This might at first sight point to the anæmia being not of hæmolytic origin. It is, however, observed that even in cases heavily infected with malaria, Van den bergh reaction may remain negative though the destruction of red blood cells un-

doubtedly occurs. Reasons against the aplastic nature of the anæmia are the high reticulocytosis and high mean corpuscular volume, both of which are low in anæmias of aplastic origin. Again, the presence of immature red cells like megaloblasts and erythroblasts in the circulating blood of these experimental monkeys point to a state of cellular regeneration of the hæmopoietic tissue. The experimental evidence therefore tends to show that the anæmia caused by administration of sulphonamide drugs is hæmolytic in origin.

The toxic effects of the drugs seem to be only temporary, as the withdrawal of the drug immediately effects a rise in the red cells, hæmoglobin and leucocytes to a level which is even higher than what is noticed at the initial stage.

Comparing the two methods of drug administration, oral and parenteral, it is seen that the latter produced a more severe type of anæmia. A large dose of the drug administered intramuscularly caused a greater degree of anæmia in a much shorter period than what was observed when the same dose was given *per os*. Blood concentration studies also revealed that in case of intramuscular injections, the drug was found to attain a much higher level of blood concentration than what was noticeable following identical doses *per os*. Apparently the sudden introduction of a large quantity of the drug causes a more pronounced damage to the hæmopoietic system.

It was also observed that sulphapyridine when administered in aqueous solution was much slower in absorption and the maximum concentration of the drug in blood was much lower than that produced by the identical dose of sulphanilamide given by the same method. This is in complete harmony with the findings of Marshall *et al* (21), and of Powell and Chen (22) who attributed it to the very low solubility of sulphapyridine as compared with sulphanilamide. Wein concluded that sulphapyridine is much less toxic for blood than sulphanilamide gram for gram. Sulphapyridine on account of its low solubility and consequent slow absorption may easily lead to this erroneous conclusion. In the present series of monkeys the degree of anæmia and reduction in the number of total leucocytes produced by the same dose of sulphapyridine were more than those produced by sulphanilamide even though blood concentration of the former is lower and the drug is slower in absorption as mentioned above. This fact indicates that sulphapyridine is more toxic than sulphanilamide at least from the hæmatological standpoint. Marshall and his associates (18) working on the lethal dose in mice, rabbits and dogs came to the same conclusion.

SUMMARY AND CONCLUSIONS

(1) A normal hæmatological standard of the different elements of blood in healthy monkeys was ascertained.

(2) The effects on the hæmopoietic system of therapeutic doses of different drugs of sulphonamide group were studied on monkeys (Silenus Rhesus) kept under controlled conditions in the laboratory.

(3) 'Prontosil Soluble' was administered by intramuscular route. 'Prontosil Album' and 'M and B. 693' were given orally through stomach tube.

(4) All the preparations used produced a definite and progressive anæmia in monkeys.

(5) Diminution of hæmoglobin and fall of erythrocyte count was found to begin between 24 and 72 hours after the commencement of the treatment and reached a maximum in 7-10 days.

(6) Mean cell volume of erythrocytes and percentage of circulating reticulocytes increased and immature red cells were found in the circulating blood of animals treated with Prontosil Soluble by injection.

(7) Total white cells decreased under Prontosil Soluble and sulphapyridine administration.

(8) Coagulation time of blood was prolonged by all the preparations.

(9) Loss of weight occurred in the animals but this loss was somewhat slower than the production of anæmia.

(10) Recovery from anæmia and from loss of weight occurred on cessation of administration of the drugs and the animals reached their normal levels in two to four weeks.

(11) Studies on concentration of the drugs in the blood showed that the maximum concentration of sulphanilamide was reached in half an hour when the drug was administered by injection and one and half hours when given by mouth.

(12) Sulphapyridine when administered in aquoeus solution orally was slower in absorption and maximum concentration of the drug in blood was much lower than that of sulphanilamide given by the same method, but when the drug was administered in acid medium absorption was much hastened and enhanced.

The above investigations were carried out by me when on leave during summer last year, at the School of Tropical Medicine, Calcutta.

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ANNEX
TABLES AND FIGURES

TABLES SHOWING HÆMATOLOGICAL FINDINGS

FIGURES SHOWING GRAPHICAL REPRESENTATIONS OF HÆMATOLOGICAL FINDINGS

FIGURES SHOWING GRAPHICAL REPRESENTATIONS
OF BLOOD CONCENTRATION FOLLOWING THE
ADMINISTRATION OF SULPHONAMIDES

TABLE I

Hæmatological Studies in Normal

Number of Animal.		Sex	Wt. in lbs.	Hæmoglobin % g. (100% = 13.75 g.)		Red blood cells per cmm.	Reticulocytes %	C.V.	M.C.V.	M.C.H.
I.	(1)	F.	8.0	90.0	12.37	5,270,000	1.0	37.0	70.2	23.5
	(2)		9.0	91.0	12.51	5,290,000	1.3	46.0	86.9	23.6
	(3)		8.5	92.0	12.65	5,260,000	2.3	41.5	78.9	24.0
	(4)		9.0	96.0	13.20	5,380,000	1.9	45.5	84.5	24.5
II.	(1)	M.	8.0	92.0	12.65	5,610,000	3.1	44.0	78.3	22.5
	(2)		8.0	94.0	12.93	5,570,000	1.1	43.0	77.1	23.2
	(3)		8.0	91.0	12.51	5,270,000	1.9	38.2	72.4	23.7
	(4)		8.5	94.0	12.93	5,370,000	1.1	41.0	76.3	24.1
III.	(1)	F.	7.5	90.0	12.38	4,800,000	1.7	38.0	79.1	25.8
	(2)		7.5	87.0	11.96	4,810,000	1.6	40.0	83.1	24.8
	(3)		8.0	90.0	12.38	5,010,000	1.2	40.0	79.8	24.7
	(4)		8.5	98.0	13.48	5,060,000	0.8	42.0	83.0	26.5
IV.	(1)	F.	7.0	95.0	13.06	5,050,000	1.0			
	(2)		7.5	94.0	12.93	4,780,000	1.9	41.0	85.7	27.0
	(3)		7.5	95.0	13.06	4,790,000	2.1	42.0	87.6	27.2
	(4)		8.5	106.0	14.58	5,020,000	1.0	44.0	87.4	29.3
V.	(1)	M.	7.0	94.0	12.93	6,030,000	0.8	37.0	61.3	21.4
	(2)		7.5	93.0	12.79	6,330,000	1.4	40.0	63.1	20.2
	(3)		8.0	95.0	13.06	5,530,000	1.3	45.0	81.3	23.6
	(4)		8.5	104.0	14.30	6,070,000	0.9	49.0	80.1	23.5
VI.	(1)	M.	7.5	92.0	12.65	5,330,000	0.8	38.0	71.3	23.7
	(2)		8.0	95.0	13.06	5,730,000	1.4	40.0	73.7	24.0
	(3)		8.0	94.0	12.93	5,360,000	0.8	46.0	85.8	24.1
	(4)		8.5	93.0	12.79	5,340,000	1.0	38.0	71.1	23.9
VII.	(1)	F.	7.0	90.0	12.38	4,810,000	2.2	41.0	85.2	25.7
	(2)		7.5	85.0	11.69	4,530,000	1.9	36.0	79.4	25.8
	(3)		8.5	91.0	12.51	5,090,000	1.8	39.0	76.6	24.5
	(4)		9.0	97.0	13.39	5,030,000	1.4	36.0	71.5	26.5
VIII.	(1)	M.	6.0	88.0	12.10	4,630,000	3.2			26.1
	(2)		6.5	94.0	12.93	5,040,000	3.5	41.0	81.3	25.6
	(3)		6.5	98.0	13.48	4,830,000	1.3	42.0	86.9	27.9
	(4)		7.0	105.0	14.48	5,310,000	0.8	44.4	83.6	27.2
IX.	(1)	F.	7.5	100.0	13.75	5,970,000	1.2	39.0	65.3	23.0
	(2)		7.5	96.0	13.20	5,230,000	1.0	42.0	80.3	25.2
	(3)		8.0	108.0	14.85	5,550,000	1.4	48.0	86.5	26.7
	(4)		9.0	120.0	16.50	5,730,000	1.2	53.0	92.4	28.7
X.	(1)	M.	8.0	108.0	14.85	6,320,000	1.4	42.0	66.4	23.5
	(2)		8.0	110.0	15.13	5,360,000	1.1	44.0	82.1	28.2
	(3)		8.5	110.0	15.13	5,510,000	0.8	42.0	76.2	27.4
	(4)		8.5	110.0	15.13	5,530,000	0.9	48.8	88.2	27.3
Mean			7.58	96.62	13.28	5,305,700	1.46	41.96	78.94	25.08
Standard Errors.			±0.02	±1.30	±1.70	±68,300	±0.11	±0.62	±1.17	±0.32

Monkeys (Silenus Rhesus) in Calcutta.

M.C.H.C.	Total Leucocytes per cmm.	Neutrophil		Lymphocyte.		Mononuclear		Eosinophil		Abnormal Cells.	Arneth count. Weighted mean.	Coagulation time in Secs.	Van den bergh.
		%	Total	%	Total	%	Total	%	Total				
33.4	13,700	48.0	6,576	50.0	6,850	2.0	274			Nil	1.81	40	-Ve
27.2	15,750	38.0	5,985	55.0	8,662	5.0	788	2.0	315	,,	1.60	30	,,
30.4	13,850	35.0	4,847	57.0	7,895	6.0	831	2.0	277	,,	1.67	35	,,
29.1	14,800	24.0	3,552	70.0	10,360	4.0	592	2.0	296	,,	1.60	35	,,
28.8	19,050	50.0	9,525	45.0	8,572	4.0	762	1.0	191	,,	1.75	45	,,
30.0	15,600	30.0	4,680	64.0	9,984	5.0	780	1.0	156	,,	1.67	40	,,
32.6	17,600									,,	—	35	,,
31.5	11,350	40.0	4,561	57.0	6,740	1.0	113	2.0	226	,,	1.46	40	,,
32.6	12,650	80.0	10,120	15.0	1,897	4.0	506	1.0	127	,,	2.06		,,
29.9	10,100	43.0	4,345	50.0	5,050	4.0	455	3.0	252	,,	1.73	30	,,
30.9	14,200	52.0	7,455	43.0	6,035	4.0	568	1.0	142	,,	1.61		,,
32.1	13,750	38.0	5,225	56.0	7,700	2.0	275	4.0	550	,,	1.32	30	,,
	8,350	60.0	5,010	36.0	3,006	3.0	250	1.0	84	,,	1.96		,,
31.5	10,300	45.0	4,635	48.0	4,893	5.0	515	2.5	257	,,	1.73		,,
31.1	11,000	49.0	5,390	46.0	5,060	4.0	440	1.0	110	,,	1.84	35	,,
33.1	5,200	39.0	2,028	58.0	3,016	1.0	52	2.0	104	,,	1.70	35	,,
34.1	19,250	42.0	8,085	50.0	9,625	7.0	1,347	1.0	193	,,	1.64		,,
31.9	13,150	44.0	5,786	51.0	6,707	4.0	526	1.0	131	,,	1.89	47	,,
29.0	13,400	36.0	4,824	55.0	7,370	7.0	1,005	2.0	804	,,	1.48	53	,,
29.1	18,400											50	,,
33.3	16,800	48.0	8,064	40.0	6,720	9.0	1,512	3.0	504	,,	1.64		,,
32.5	14,900	45.0	6,705	50.0	7,450	3.0	372	2.0	372	,,	1.65		,,
28.1	14,800												,,
33.7	16,550	35.0	5,792	63.0	10,427	2.0	331			,,	1.51	45	,,
30.1	8,650	50.0	4,325	43.0	3,719	4.0	346	3.0	260	,,	1.55		,,
32.5	10,000	44.0	4,400	47.0	4,700	7.0	700	2.0	200	,,	1.73	47	,,
32.1	13,600	55.0	7,480	39.0	5,304	3.0	408	3.0	408	,,	1.85	53	,,
37.1	13,950	42.0	5,859	53.0	7,394	2.0	279	3.0	418	,,	1.49	50	,,
	15,950	60.0	9,570	34.0	5,423	6.0	957			,,	2.0	40	,,
31.5	14,300	33.0	4,648	62.0	8,937	4.0	572	1.0	143	,,	1.81		,,
32.0	19,050	28.0	5,334	67.0	12,760	3.0	572	2.0	382	,,	1.55	40	,,
32.5	19,900	43.0	8,557	51.0	10,149	3.0	597	3.0	597	,,	1.50	40	,,
35.2	12,750	45.0	5,737	49.0	6,248	3.0	383	3.0	383	,,	1.50		,,
31.4	14,600	28.0	4,088	68.0	9,928	2.0	292	2.0		,,	1.62		,,
30.9	16,850	43.0	7,246	47.0	7,919	3.0	506	7.0	1179	,,	1.39		Hæm
31.1	13,100	40.0	5,240	53.0	6,943	3.0	393	4.0	524	,,	1.47	60	,,
35.3	11,650	21.0	2,447	72.0	8,388	5.0	583	2.0	232	,,	1.5		-Ve.
34.3	7,200	26.0	1,872	70.0	5,040	3.0	216	1.0	72	,,	1.74		,,
36.0	9,050	36.0	3,458	60.0	5,430	3.0	272	1.0	90	,,	1.72	50	,,
31.1	12,450	29.0	3,610	65.0	8,092	3.0	374	3.0	374	,,	1.54	40	,,
31.82	13,684	41.73	5,595	52.4	7,038	3.9	534	2.2	313	,,	1.66	42	,,
±0.35	±542.9	±1.87	±331.5	±1.91	±391.9	±0.29	±51.15	±0.21	±39.03	,,	±0.07	±1.60	

TABLE II

GROUP I. *Animal No. 1 (Female), (Body wt. 9 lbs.)*

0.06 g. per

Date.	Wt. in lbs.	Drug given.		Hæmoglobin %.	Hæmoglobin in g.	R.b.c. per cmm. in millions.	Reticulo %.	C.V.	M.C.V.	M.C.H.	M.C.H.C.	Total W.B.C. per cmm.
25.7.40	8.			90	12.37	5.27	1.0	37.0	70.20	23.48	33.44	13,700
1.8.40	9.			91	12.51	5.29	1.3	46.0	86.9	23.6	27.2	15,750
7.8.40	8.5			92	12.65	5.26	2.3	41.5	78.9	24.0	30.4	13,850
15.8.40	9.	Blood taken at 10-50 A.M.		96	13.20	5.38	1.9	45.5	84.5	24.5	29.1	14.800
Mean	8.63			92	12.68	5.275	1.63	42.5	80.125	23.89	30.04	14,525
16.8.40		Prontosil soluble (5% Soln.) I.M. Inj. at 11 A.M.										
17.8.40		Blood taken at 10-50 A.M. Inj. Prontosil 5 cc. I.M. at 11 A.M.		95	13.062	5.24	2.0	40.0	76.3	24.8	32.6	13,150
18.8.40		Do.										
19.8.40		Blood taken (Inj. 5 cc. given).		87	11.962	5.17	2.6	41.0	79.3	23 1	29.2	14,600
20.8.40		Do	5th Inj.	88	12.100	4.96	2.1	37.0	74.6	24.3	32.7	11,600
21.8.40		,,	6th ,,	81	11.137	4.57	3.4	37.0	80.9	24.3	30.1	10,300
22 8.40		,,	7th ,,	78	10.725	4.28	3.5	36.0	84.1	25.1	29.7	10,750
23.8.40	8.5	,,	8th ,,	74	10.175	4.19	3.5	34.0	81.1	24.2	29.9	9,900
24.8.40		,,	9th ,,	74	10.175	4.37	3.9	35.0	80.0	23.2	29.1	9,800
25 8.40		,,	10th ,,									
26.8.40		,,	11th ,,	80	11.000	4.37	3.0	35.8	81.9	25.1	30.7	11,650
27 8.40		,	12th ,,	79	10.862	4.10	3.4	35.0	85.3	26.5	31.1	10,250
28.8.40		,,	13th ,,	76	10.450	3.89	6.2	34.0	87.4	26.8	30.7	10,350
29.8.40		,,	14th ,,	78	10.725	4.24	5.4	34.8	82.1	25.3	30.8	7.050
30.8.40		,,	15th ,,	75	10.312	4.05	8.5	36.5	88.8	25.4	28.6	8,100
31.8.40		,,	16th ,,	79	10.862	4.33	5.8	34.0	78.4	25.1	31.9	6,050
1.9.40		,,	17th ,,									

SOLUBLE

kilo body-weight

0.25 g. daily by intramuscular injection for 20 days.

Neutrophils.		Lymphocytes.		Large Monos.		Eosinophils.		Basophils.		Abnormal Cells.	Arneth Count.	Van den bergh.	Coagulation time in secs.
%	Total	%	Total	%	Total	%	Total	%	Total				
48	6,576	50	6,850	2	274					Nil	1.81	Negative	40
38	5,985	55	8,662	5	788					,,	1.60	,,	30
35	4,847	57	7,895	6	831	2	277			,,	1.67	,,	35
24	3,552	70	10,360	4	592	2	296			,,	1.60	,,	35.
36.3	5,240	58	8,442	4.3	621.3	1.50	222			Nil	1.67	Negative	35
41	5,392	55	7,232	2	264	1	131	1	131	Nil	1.67	Negative	
50	7,300	45	6,570	3	438	1	146	1	146	,,	1.49	,,	
40	4,640	57	6,612	3	348					,,	1.77	Not done	
27	2,781	69	7,107	2	206	1	103	1	103	,,	2,18	Negative	
49	5,268	46	4,945	3	323	1	107	1	107	,,	1.63	Hæmo-lysis	
40	3,960	56	5,544	2	198	1	99	1	99	,,	1.53	,,	
39	3,822	58	5,684	1	98	1	98	1	98	,,	1.54	,,	
36	3,690	61	6,253	2	205	1	102			V + P + A +	1.68	Negative	
39	4,036	55	5,693	2	207	3	311	1	103	V + P +	1.66	Hæm. +	
33	2,326	60	4,230	3	212	4	282			N + V +	1.66	Negative Negative	
41	2,480	54	3,267	2	121	3	182			Norm. +	1.73	Hæm. + Erythro-blast	

TABLE II (*Contd.*)

GROUP I. *Animal No. 1 (Female) Contd.*

Date.	Wt. in lbs.	Drug given.	Hæmoglobin %.	Hæmoglobin in g.	R.b.c. per cmm. in millions.	Reticulo %.	C.V.	M.C.V.	M.C.H.	M.C.H.C.	Total W.B.C. per cmm.
		Blood taken. (Inj. 5 cc. given).									
2.9.40	8.	Do. 18th Inj.	76	10.450	4.38	5.2	37.5	85.6	23.8	27.8	7,100
3.9.40		,, 19th ,,	74	10.175	4.12	4.3	35.0	84.9	24.6	29.1	8.050
4.9.40		,, 20th ,,	80	11.000	4.31	4.8	37.0	85.8	25.5	29.7	10,400
5.9.40		Blood taken.	73	10.037	4.06	5.3	37.0	91.1	24.7	27.3	5,950
11.9.40		Blood taken (a week after stoppage of drug).	96	13.200	4.84	1.7	46.0	95.0	27.2	28.7	9,650
18.9.40	9.	,, (2 wks. ,,)	106	14.575	5.72	1.3	47.0	82.2	25.4	31.0	10,700
25.9.40		,, (3 wks. ,,)	108	14.850	5.38	1.2	42.0	78.1	27.6	35.3	12,300
2.10.40	10.	,, (4 wks. ,,)	102	14.025	5.58	0.8	48.0	86.0	25.1	29.2	13,050
		(Second Course).									
2.10.40		Prontosil by I.M. Inj. 5cc. 5% Soln.									
3.10.40		Prontosil Inj. Blood examn.	92	12.650	4.67	1.1	40.9	85.5	27.1	30.9	11,000
4.10.40		3rd Inj. Do.	87	11.962	4.31	0.8	40.0	92.8	27.7	29.9	10,650
5.10.40	9.	Blood Examn.	80	11.000	4.98	1.4	38.0	76.3	22.1	26.3	7,700
14.10.40	10.	Blood a wk. after stoppage of drug.	90	12.375	5.02	1.2	41.0	81.6	24.6	30.1	9,500

Neutrophils.		Lymphocytes.		Large Monos.		Eosinophils.		Basophils.		Abnormal Cells.	Arneth Count.	Van den bergh.	Coagulation time in secs.
%	Total	%	Total	%	Total	%	Total	%	Total				
43	3,053	55	3,905	1	71	1	71			Norm. +	1.68	Hæm. +	
40	4,160	55	5,720	1.	104	4	416				1.68	Negative	
32	1,904	64	3,808	2	119	2	119				1.79	Hæmo-lysis	
30	2,895	64	6,177	3	289	3	289				1.72	Negative	
28	2,996	63	6,741	5	535	4	428				1.69	Hæm. +	
33	4,059	63	7,749	2	246	2	246			Nil.	1.58	,, -Ve.	30.
												Hæm. +	
41	4,367	54	5,751	2	213	3	319			Nil.	1.50	-Ve. -Ve.	55.
24	2,280	69	6,555	3	285	4	380			Nil.	1.36	Hæm. +	30.

TABLE III

PRONTOSIL

GROUP I.   *Animal No. 3. (Body wt. 8½ lbs.)*

(0.06 g. per

Date.	Wt. in lbs.	Drugs given.	Hæmoglobin %.	Hæmoglobin in g.	R.b.c. per cmm. in millions.	Reticulo %.	C.V.	M.C.V.	M.C.H.	M.C.H.C.	Total W.B.C. per cmm.
27.7.40	7.5		90	12.37	4.800	1.7	38.0	79.1	25.78	32.56	12,650
2.8.40	7.5		87	11.96	4.810	1.6	40.0	83.1	24.8	29.9	10,100
8.8.40	8		90	12.37	5.010	1.2	40.0	79.8	24.7	30.9	14,200
23.8.40	8.5		98	13.47	5.060	0.8	42.0	83.0,	26.5	32.1	13,750
Mean	7.87		91.02	12.54	4.920	1.3	40.0	81.25	25.445	31.36	12,650
23.8.40		1st Inj. Pronto-sil (I.M.) given 5 cc. of 5% Soln.									
24.8.40		2nd do	90	12.37	4.570	2.1	40.0	87.7	26.8	30.9	12,350
25.8.40		3rd do									
26.8.40		4th do Blood taken before Inj.	80	11.00	3.800	2.1	38.2	105.3	26.3	29.9	11,450
27.8.40		5th do	78	10.72	3.790	2.1	38.0	100.2	28.3	29.0	10,250
28.8.40		6th do	72	9.90	4.010	3.4	36.0	89.7	24.7	27.5	10,250
29.8.40		7th do	77	10.58	4.050	4.3	35.0	86.4	26.1	30.2	6,950
30.8.40		8th do	77	10.58	3.860	3.9	35.0	90.7	27.2	30.2	10,450
31.8.40		9th do	71	9.76	3.560	6.3	33.3	93.5	27.4	29.3	8,200
1.9.40		10th do									
2.9.40	8	11th do	72	9.90	3.140	3.6	35.0	111.4	31.5	28.3	9,900
3.9.40		12th do	76	10.45	3.990	5.2	38.0	95.2	26.2	27.5	7,500
4.9.40		13th do	78	10.72	3.970	5.1	38.0	95.7	27.1	28.2	8,100
5.9.40		14th do	82	11.27	4.020	4.4	38.0	94.5	28.0	29.6	9,650
6.9.40	7.5	15th do	85	11.68	3.880	3.2	40.2	103.6	30.1	29.1	9,050
7.9.40		Blood taken.	83	11.41	3.880	3.1	39.0	100.5	29.4	29.3	7,050
13.9.40	9	Blood a wk. after stoppage of drug.	95	13.06	4.520	0.9	46.0	101.7	28.9	32.6	9,300
20.9.40	9.5	Blood 2 wks. do	102	14.02	4.710	0.7	47.0	99.8	29.7	29.8	14,450
27.9.40	9.5	Blood 3 wks. do	100	13.75	4.840	0.7	50.0	103.3	26.3	27.5	11,150
7.10.40	10.5	Blood 4 wks. do	112	15.40	5.320	0.5	50.0	93.9	28.9	30.8	10,150

SOLUBLE

kilo body weight). *0.25 g. daily by I. M. Inj. for 15 days.*

Neutrophils.		Lymphocytes.		Large Monos.		Eosinophils.		Basophils.		Abnormal Cells.	Arneth Count.	Van den bergh.	Coagulation time in secs.
%	Total	%	Total	%	Total	%	Total	%	Total				
80	10,120	15	1,897	4	506	1	127			Nil	2.06	Negative	
43	4,343	50	5,050	4	455	3	252			,,	1.73	,,	30
52	7,455	43	6,035	4	568	1	142			,,	1.61	,,	
38	5,225	56	7,700	2	275	4	550			,,	1.32	Hæm. +	30
53.1	6,785	41	5,170	3.5	451	2.25	257.5			Nil	1.68	Negative	30
55	6,793	37	4,569	5	617	3	371			Nil	1.42	Negative	90
46	5,268	50	5,725	1	114	2	229	1.0	117	,,	2.02	Hæm. +	
43	4,408	51	5,227	2	205	3	308	1.0	102	Norm. + V + P +	1.60	,,	
40	4,100	55	5,637	2	205	3	308			V + P +	1.50	,,	
36	2,502	56	3,892	4	278	4	278			Norm. + Erythro-blast +	2	,,	
45	4,702	49	5,121	4	418	2	209			Norm. +	1.66	Negative	45
51	4,182	44	3,608	3	246	2	164			Erythro. + do	1.50	,,	
45	4,455	48	4,752	4	396	2	198			Norm. +	1.33	Hæm. –	60
40	3,000	57	4,275	1	75	2	150			,,	1.61	,,	
40	3,240	53	4,293	2	162	4	324	1	81	Nil	1.61	Negative	50
52	5,018	41	3,957	2	193	5	482			,,	1.56	Negative	
43	3,892	51	4,615	4	362	2	181			,,	1.61	,,	
45	3,375	51	3,825	1	75	3	225			,,	1.45	Hæm. +	50
												Negative	50
36	5,202	56	8,092	4	578	4	578			Nil	1.48	Negative	
33	3,679	60	6,690	3	335	4	446			,,	1.29	Hæm. +	
												Negative	50

·4·

TABLE IV

GROUP I. *Animal No. 4. Male (9 lbs. body wt.)*

PRONTOSIL

0.9 g. per

Date.	Wt. in lbs.	Drug given.	Haemoglobin %.	Haemoglobin in g.	R.b.c. per cmm. in millions.	Reticulo %.	C.V.	M.C.V.	M.C.H.	M.C.H.C.	Total W.B.C. per cmm.
27.7.40	7		95	13.06	5.050	I			25.86		8,350
2.8.40	7.5		94	12.92	4.780	1.9	41	85.7	27.0	31.5	10,300
8.8.40	7.5		95	13.06	4.790	2.1	42	87.6	27.2	31.1	11,000
9.9 40	8.5	Blood taken.	106	14.57	5.020	1.0	44	87.4	29.3	33.1	5,200
Mean	7.62		97.5	13.40	4.910	1.5	42.3	86.9	27.34	31.9	8,713
9.9.40		Prontosil Inj. 0.375-grs. given Blood taken 2 hr. after Inj.	103	14.16	4.840	1.3					20,700
10.9.40		do. Blood before Inj.	92	12.65	4.110	2.2	41	99.7	30.7	30.9	6,200
11.9.40		do. do.	80	11.00	3.830	2.8	34	88.7	28.7	32.3	9,100
12.9 40		do. (4th Inj.)	78	10.72	3.430	1.6	36.6	106.7	31.6	29.6	11,100
13 9.40		do. (5th Inj.)	74	10.17	3.720	5.4	34.0	91.7	27.3	29.9	5,850
		Blood 2 hrs. after Inj.	73.	10.03	3.610		31.0	85.8	27.8	32.3	8,200
14.9 40		do. (6th Inj.) Blood.	68	9.35	3.570	6.3	31.0	86.8	26.2	31.5	5,450
15.9.40		do. (7th Inj.)									
16.9.40		Blood.	73	10.03	3.540	15.0	38 0	107.3	28.3	26.4	5,650
23.9.40	S	Blood a week after stoppage of drug.	80	11.00	4 900	2.0	41.0	83.7	22.4	26.8	7,600
1.10.40	9	Blood 2 wks. do.	108	14.85	5.450	1.0	47.0	86.2	27.2	31.3	8,150
15.10.40	9	Blood 3 do.	110	15.12	5.250	0.6	47.5	90.4	28.8	31.9	11,250
22.10.40	9	Blood 4 do.	109	14.98	5.640	0.9	49.0	86.8	26.5	30.5	7,000

SOLUBLE
kilo body weight

0.375 g. daily by I. M. Inj. for 7 days

Neutrophils.		Lymphocytes.		Large Monos.		Eosinophils.		Basophils.		Abnormal Cells.	Arneth Count.	Van den bergh.	Coagulation time in secs.
%	Total	%	Total	%	Total	%	Total	%	Total				
60	5,010	36	3,006	3	250	1	84			Nil	1.96	Negative	
45	4,635	48	4,893	5	515	2.5	257			,,	1.73	Negative	
49	5,390	46	5,060	4	440	1	110			,,	1.84	,,	35
39	2,028	58	3,016	1	52	2	104			,,	1.70	Hæm. +	35
48.2	4,266	47	3,973	3.25	314	1.62	138.7			Nil	1.80	Negative	35
58	3,596	35	2,170	3	186	3	186	1.0	62	Nil	2.08	Hæm. +	
												Negative	
58	6,438	39	4,329	1	111	2	222			,,	1.79	Negative	
												Hæm. +	
35	1,908	60	3,270	2	109	3	163			Norm + Eryth. +	2.16	Negative	
										P + A +			
27	1,526	70	3,955	3	169					Norm. + Eryth. +	1.86	Negative	60
38	2,888	58	4,408	2	152	2	152			Nil	1.69	Negative	
28	2,282	65	5,298	2	163	5	407			,,	1.73	Negative	
35	3,938	60	6,750	2	225	3	337			,,	1.58	Hæm. +	35
31	2,170	64	4,480	1	70	4	280			,,	1.47	Negative	45

TABLE V

PRONTOSIL

GROUP II. *Animal No. 5. Male (8½ lbs. body wt.)*   0.06 g. per

Date.	Wt. in lbs.	Drugs given.	Hæmoglobin %.	Hæmoglobin in g.	R.b.c. per cmm. in millions.	Reticulo %.	C.V.	M.C.V.	M.C.H.	M.C.H.C.	Total W.B.C. per cmm.
30.7.40	7		94	12.92	6.030	0.8	37.0	61.3	21.4	34.09	19,250
5.8.40	7.5		93	12.78	6.330	1.4	40.0	63.1	20.2	31.0	13,150
9.8.40	8		95	13.06	5.530	1.3	45.0	81.3	23.6	29.0	13,400
3.9.40	8.5		104	14.30	6.070	0.9	49.0	80.07	23.5	29.1	18,400
Mean	7.75		96.5	13.26	5.990	1.1	42.75	71.44	22.17	31.02	16,050
3.9.40		Prontosil album 0.25 grm. given dissolved in 5 ccs. of tap water by stomach tube.									
4.9.40		do.									
5.9.40		do.									
6.9.40		do. 0.242 do.	100	13.75	5.380	1.0	43.0	79.9	25.5	31.9	15,550
7.9.40		(5th day) do. Blood before Inj. Diarrhœa +									
8.9.40		Prontosil alb. do.									
9.9.40		do.									
10.9.40		do.									
11.9.40		do. (9th day)	87	11.96	5.110	4.5	43.0	84.1	23.4	27.8	11,150
12.9.40		,, (10th ,,)									
13.9.40		,, (11th ,,)									
14.9.40		,, (12th ,,)									
16.9.40		,, (13th ,,)	84	11.55	5.140		40.0	77.8	22.8	28.8	12,600
17.9.40		,, (14th ,,)									
18.9.40		,, (15th ,,)									
19.9.40	6	,, (16th ,,)	90	12.37	5.070	2.5	42.0	82.8	24.4	29.4	8,950
20.9.40		,, (17th ,,)									
21.9.40		,, (18th ,,)									
22.9.40		,, (19th ,,)									
23.9.40		,, (20th ,,)									
25.9.40	5.5	Blood.	85	11.68	5.160	1.4	40.0	77.5	22.6	29.8	13,000
2.10.40	6.5	Blood a week after stoppage of drug.	96	13.20	5.560	1.0	47.0	84.5	23.7	28.1	10,900
10.10.40		do. 2 wks.	100	13.75	5.700	0.9	44.0	77.2	24.1	31.2	7,550
17.10.40	7	do. 3 wks.	100	13.75	5.730	0.5	43.4	75.7	23.9	31.6	9,900

ALBUM AS SUSPENSION IN WATER

kilo body weight   *0.242 g. daily for 20 days given by stomach tube.*

Neutrophils.		Lymphocytes.		Large Monos.		Eosinophils.		Basophils.		Abnormal Cells.	Arneth Count.	Van den bergh.	Coagulation time in secs.
%	Total	%	Total	%	Total	%	Total	%	Total				
42	8,085	50	9,625	7.0	1,347	1	193			Nil	1.64	Neg	
44	5,786	51	6,707	4.0	526	1	131			Nil	1.89	,,	47
36	4,824	55	7,370	7.0	1,005	2	804			Nil	1.48	,,	53
												,,	50
40.63	6,231	52	7,900	6.0	959	1.33	174.3			Nil	1.67	,,	50
30	4,665	64	9,952	3.0	466	2	311	1	156	V+	1.73	Haem+	
												Neg.	50
34	4,284	64	8,064	2.0	252					Nil	1.63	Neg.	45
29	2,596	68	6,087	1.0	89	1	89	1	89	Nil	1.64	Neg.	
40	5,200	57	7,410	3.0	390					Nil	1.43	Haem+	
												Neg.	40
36.0	2,718	61	4,605	3.0	2,265					Nil	1.44	Haem+	
39.0	3,861	58	5,742	1.0	99	2	198			Nil	1.47	,,	

TABLE VI

GROUP II. *Animal No. 7, Female (9 lbs. body wt.)*

PRONTOSIL

0.06 g. per

Date.	Wt. in lbs.	Drug given.	Haemoglobin %.	Haemoglobin in g.	R.b.c. per cmm. in millions.	Reticulo %.	C.V.	M.C.V.	M.C.H.	M.C.H.C.	Total W.B.C. per cmm.
31.7.40	7.0		90	12.37	4.810	2.2	41	85.2	25.7	30.1	8,640
6.8.40	7.8		85	11.68	4.530	1.9	36	79.4	25.8	32.5	10,000
12.8	8.5		91	12.51	5.090	1.8	39	76.6	24.5	32.1	13,600
5.9	9.0		97	13.33	5.030	1.4	36	71.5	26.5	37.1	13,950
Mean	8.0		90.7	12.39	4.865	1.82	38	78.17	25.6	32.9	11,550
5.9.40		Prontosil 0.256 grm. by *stomach tube.*									
		do. Blood taken before drug.	80.0	11.00	4.030	1.8	38	94.2	27.3	28.9	16,050
7.9		do.									
8.9		do.									
9.9		do. 5th day do.	76	10.45	4.300	4.5	38	88.3	23.8	27.5	14,900
10.9		,,									
11.9		,,									
12.9		,, ,,	75	10.31	3.710	2.9	40	118.6	27.7	25.8	14,700
13.9		,,									
14.9											
16.9	8.5	11th day ,,	83	11.41	2.950	4.2	40	135.6	38.7	28.5	12,400
17.9.		12th ,, ,,									
18.9		13th ,, ,,									
19.9.		14th ,, ,,	85	11.69	4.250	2.1	45	105.9	27.4	25.9	16,450
20.9.		15th ,, ,,									
21.9.		16th ,, ,,									
22.9.		17th ,, ,,									
23.9		18th ,, ,,	85	11.68	4.580	2.1	39	85.1	25.5	29.9	14,550
24.9.		19th ,, ,,									
25.9		20th ,, ,,									
26.9	8	Blood	84	11.55	4.970	2.3	42	84.5	23.2	27.3	15,950
3.10.40	9	Blood exam. a	94	12.92	5.410	0.7	41	75.7	23.8	31.5	18,250
25.9.40 26.9.40	8	wk. after stoppage of drug.									
16.10.40	10	Blood.	98	13.47	5.340	1.1	48	89.8	25.2	28.1	15,250

ALBUM AS SUSPENSION IN WATER

kilo body weight   *0.256 g. daily for 20 days given by stomach tube.*

Neutrophils.		Lymphocytes.		Large Monos.		Eosinophils.		Basophils.		Abnormal Cells.	Arneth Count.	Van den bergh.	Coagulation time in secs.
%	Total	%	Total	%	Total	%	Total	%	Total				
50	4,325	43	3,719	4	346					Nil	1.55	Negative	
44	4,400	47	4,700	2	200					Nil	1.73	Negative	47
55	7,480	39	5,304	3	408	3	408			Nil	1.85	,,	53
42	5,859	53	7,394	2	279	3	418			Nil	1.49	,,	50
47.7	5,516	45.5	5,279	4	433	2.8	321			Nil	1.65	,,	50
50	8,025	46	7,383	2	321	2	321			Nil	1.65	Hæm.+	
44	6,556	49	7,301	3	447	4	596			Nil	1.57	Negative.	
44	6,468	50	7,350	2	294	4	588			A+ P+	1.56	Hæm.+	
36	4,464	56	6,944	3	372	5	620				1.84	Negative.	
44	7,238	48	7,896	3	493	5	823			Nil	1.56		
35	5,092	59	8,585	2	291	3	436	1	146	Nil	1.46	Negative.	
53	8,453	41	6,595	2	319	4	638			Nil	1.62	Negative	70
50	9,125	45	8,212	2	365	3	548			Nil	1.45	Negative	70
38	5,795	56	8,540	2	305	4	610			Nil	1.31	Negative	55

TABLE VII

GROUP II.   *Animal No. 8, Male (7 lbs. body wt.)*   0.09 g. per

Date.	Wt. in lbs.	Drug given.	Haemoglobin %.	Haemoglobin in g.	R.b.c. per cmm. in millions.	Reticulo %.	C.V.	M.C.V.	M.C.H.	M.C.H.C	Total W.B.C. per cmm.
31.7.40	6		88	12.10	4.630	3.2			26.1		15,950
6.8.40	6.5		94	12.92	5.040	3.5	41.0	81.3	25.6	31.5	14,300
12.8.40	6.5		98	13.47	4.830	1.3	42.0	86.9	27.9	32.0	19,050
17.9.40	7.0		105	14.43	5.310	0.8	44.4	83.6	27.2	32.5	19,900
Mean	6.5		96.2	13.23	4.952	2.2	42.4	83.9	26.7	32.0	17,300
17.9.40	7.0	Prontosil album 0.3 grms. by stomach tube.									
18.9.40		do.									
19.9.40		do.									
20.9.40		4th day ,, Blood	87	11.96	4.120	3.2	39.0	94.7	29.1	30.6	23,550
21.9.40		5th ,, ,, slight diarrhœa									
22.9.40		6th ,, ,,									
23.9.40		7th ,, ,,									
24.9.40	6.5	8th ,, ,, Blood before drug	92	12.65	4.640	1.4	43.1	92.8	27.2	29.3	10,250
27.9.40		9th ,, ,,									
28.9.40		10th ,, ,,									
29.9.40		11th ,, ,,									
30.9.40	6.0	12th ,, ,, Blood	79	10.86	4.390	2.2	38.0	86.5	24.7	28.6	13,450
1.10.40		13th ,, ,,									
2.10.40		14th ,, ,,									
3.10.40		15th ,, ,,									
4.10.40		16th ,, ,,									
5.10.40		17th ,, ,,									
6.10.40		18th ,, ,,									
7.10.40		19th ,, ,,									
8.10.40		20th ,, ,,									
9.10.40	5.0	Blood.	80	11.00	4.400	2.2	41.0	93.1	25.0	26.8	13,500
16.10.40	6	Blood exam. a wk. after stoppage of drug.	102.0	14.02	5.110	1.1	45.5	89.0	27.4	30.8	13,050

AS SUSPENSION IN WATER

kilo body weight   *0.3 g. daily for 20 days given by stomach tube.*

| Neutrophils. | | Lymphocytes. | | Large Monos. | | Eosinophils. | | Basophils. | | Abnormal Cells. | Arneth Count. | Van den bergh. | Coagulation time in secs |
%	Total	%	Total	%	Total	%	Total	%	Total				
60	9,570	34	5,423	6	957					Nil	2.0	Negative	40
33	4,648	62	8,937	4	572	1	143			,,	1.81	Negative	
28	5,334	67	12,763	3	572	2	382			,,	1.55	Negative	40
43	8,557	51	10,149	3	597	3	597			,,	1.50	Negative	40
41	7,027	53.5	9,318	4	674	1.5	280.5			Nil	1.715	Negative	40
55	12,952	39	9,185	3	706	3	706			Nil	1.74	Hæm. +	45
32	3,280	63	6,457	3	308	2	205			. ,,	1.42	Negative	
48	6,456	49	6,590	2	269	1	135				1.63	Hæm. --	
52	7,020	44	5,940	2	270	2	270			,,	1.96	Hæm. +	75
40	5,220	57	7,438	1	131	2	261			Nil	1.40	Hæm. --	38

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TABLE VIII

M. & B. 693 finely powdered

GROUP III.   *Animal No. 10, Male (Weight 8½ lbs.)*   0.06 g. per

Date.	Wt. in lbs.	Drug given.	Hæmoglobin %.	Hæmoglobin in g.	R.b.c. per cmm. in millions.	Reticulo %.	C.V.	M.C.V.	M.C.H.	M.C.H.C.	Total W.B.C. per cmm.
13.8.40	8		108	14.85	6.320	1.4	42.0	66.4	23.5	35.3	11,650
20.8.40	8.0		110	15.12	5.360	1.1	44.0	82.1	28.2	34.3	7,200
2.9.40	8.5		110	15.12	5.510	0.8	42.0	76.2	27.4	36.0	9,050
27.9.40	8.5		110	15.12	5.530	0.9	48.8	88.2	27.3	31.1	12,450
27.9.40		(1st day) M.B. 693 per os. thro. stomach tube 0.242 grms.									
28.9.40		do.									
29.9.40		do.									
30.9.40		4th day ,, Blood	83	11.41	4.440	1.0	39.0	87.8	25.7	29.2	8,200
1.10.40		,,									
2.10.40		,,									
3.10.40		,,									
4.10.40		8th ,, ,, Blood	82	11.27	3.630	3.2	41.9	112.7	31.1	25.9	6,050
5.10.40		,,									
6.10.40		,,									
7.10.40		,,									
8.10.40		,,									
9.10.40	8.0	13th ,, ,, Blood	80.0	11.00	4.640	3.5	40.0	86.2	23.7	27.5	8,200
10.10.40		14th ,, ,,									
11.10.40		15th ,, ,,									
12.10.40		16th ,, ,, Blood	85	11.68	4.310	5.2	40.0	92.8	27.1	29.2	9,100
13.10.40		17th ,, ,,									
14.10.40		18th ,, ,,									
15.10.40	7.0	19th ,, ,,									
16.10.40	6.0	20th ,, ,,									
17.10.40		*Blood.*	83	11.71	4.480	2.5	41.0	91.5	25.4	27.5	6,400
24.10.40		Blood exam. a wk. after stoppage of drug.	100	13.75	4.930	1.3	42.0	85.1	27.8	32.7	9,300

and in tap water suspended

kilo body weight *0.242 g. daily for 20 days given by stomach tube.*

Neutrophils		Lymphocytes.		Large	Monos.	Eosinophils.		Basophils		Abnormal Cells.	Arneth Count.	Van den bergh.	Coagulation time in secs.
%	Total	%	Total	%	Total	%	Total	%	Total				
21	2,447	72	8,388	5	583	2	232			Nil	1.5	Negative	
26	1,872	70	5,040	3	216	1	72			,,	1.74	,,	
36	3,458	60	5,430	3	272	1	90			,,	1.72	,,	50
29	3,610	65	8,092	3	374	3	374			,,	1.54	,,	40
66	5,412	32	2,624	2	164					Nil	1.72		80
36	2,178	60	3,630	1	61	3	181			,,	1.98	Negative	
												Hæm. +	90
52	4,732	45	4,095	1	91	2	182			,,	1.43	Negative	
51	3,264	45	2,880	2	128	2	128			,,	1.73	Hæm. +	86
50	4,650	45	4,185	3	279	2	186			Nil	1.47	Negative	

TABLE IX

GROUP III.   *Animal No. 12, (Weight 9 lbs.)*

Date.	Wt. in lbs.	Drug given.	Hæmoglobin %.	Hæmoglobin in g.	R.b.c. per cmm. in millions.	Reticulo %.	C.V.	M.C.V.	M.C.H.	M.C.H.C.	Total W.B.C. per cmm.
1.10.40			108	14.85	5.450	1.0	47	86.2	27.2	31.3	8,150
15.10.40			110	15.12	5.250	0.6	47.5	90.4	28.8	31.9	11,250
22.10.40	9		109	14.98	5.640	0.9	49.0	86.8	26.5	30.5	
Mean			109	14.93	5.446	0.83	47.83	87.8	27.5	31.2	9,700
22.10.40		1st. day MB 693 0.257 grms. per os.									
23.10.40		2nd day do.									
24.10.40		3rd ,, ,,									
25.10.40	8	4th ,, ,, Blood.	100	13.75	4.500	1.3	39.2	87.1	30.5	35.0	7,100
26.10.40		5th ,, do.									
27.10.40		6th ,, ,,									
28.10.40	7 5	7th ,, ,, Blood.	96	13.20	4.450	3.5	40.5	91.0	29.6	32.5	4,600

suspended in tap water

kilo body weight   *0.257 g. daily for 7 days given by stomach tube.*

Neutrophils.		Lymphocytes.		Large Monos.		Eosinophils.		Basophils.		Abnormal Cells.	Arneth Count.	Van den bergh.	Coagulation time in secs.
%	Total	%	Total	%	Total	%	Total	%	Total				
28	2,282	65	5,298	2	163	5	407			Nil	1.75	Negative	
35	3,938	60	6,750	2	225	3	337			Nil	1.58	Hæm. +	35
31	2,170	64	4,480	1	70	4	280			Nil	1.47	Negative	45
											1.59		40
45	3,195	51	3,621	1	71	3	213			Nil	1.79	Hæm.	
40	1,840	53	2,438	2	92	5	230			Nil	1.46	Hæm. +	50

TABLE X

GROUP IV.   *Animal No. 11, (9 lbs. body wt.)*

Date.	Wt. in lbs.	Drug given	Hæmoglobin %	Hæmoglobin in g.	R.b.cs per cmm. in millions.	Reticulo %	C.V.	M.C.V.	M.C.H.	M.C.H.C.	Total W.B.C. per cmm.
21.9.40	8.5		99	13 61	5.640	1.2	43.0	76.2	24.1	31.6	14,950
4.10.40	9.0	M.B. 693 dissolved in HCl. acid per os. stomach tube.	110	15.12	5.870	1.0	44.0	74.9	25.7	34.3	15,250
5.10.40		do.									
6.10.40		do.									
7.10.40		(4th day) do. Blood.	77	10.58	3.980	3.0	36.4	91.4	26.6	29.1	9,950
8.10.40		M.B. 693 0.256 grms. per os.									
9.10.40		do.									
10.10.40		(7th day) do.	73	10.03	3.950	4.0	38.9	98.4	25.4	25.8	8,750
11.10.40		(8th ,,) ,,									
12.10.40		(9th ,,) ,,									
13.10.40		(10th ,,) ,,									
14.10.40	8.0	(11th ,,) ,, Blood.	85	11.68	4.530	5.3	42.0	92.7	25.8	27.8	7,400
15.10.40		(12th ,,) do.									
16.10.40		(13th ,,) ,,									
17.10.40		(14th ,,) ,,									
18.10.40	7.5	(15th ,,) ,, Blood.	77	10.58	4.200	2.7	36.5	86.9	25.2	29.4	9,800
19.10.40		(16th ,,) do.									
20.10.40		(17th ,,) ,,									
21.10.40		(18th ,,) ,, Blood.	80	11.00	4.150	5 2	37.0	86.7	26.5	29.8	7,600
22.10.40		(19th ,,) do.									
23.10.40		(20th ,,) ,,									
24.10.40		Blood exam.	72	9.90	4.140	3.8	39	94.2	23.9	25.3	8,000
29.10.40	8.5	Blood exam. a wk. after stoppage of drug.	100	13.75	4.730	1.3	40.0	84.5	29.1	34.4	10,100

dilute Hydrochloric Acid

kilo body weight   *0.256 g. daily for 20 days given by stomach tube.*

Neutrophils.		Lymphocytes.		Large Monos.		Eósinophils.		Basophils.		Abnormal Cells	Arneth Count	Vanden Bergh	Coagulation time Secs.
%	Total	%	Total	%	Total	%	Total	%	Total				
20	2,990	74	11,065	4	598	2	299			Nil	1.85	Negative	40
29	4,422	67	10,218	2	305	2	305			,,	1.35	,,	40
												Hæm. +	
29	2,537	64	5,600	2	175	5	438			Norm. +	1.51	Negative	
												Negative	
37	3,626	55	5,390	3	294	5	490			Nil	1.90	Negative	100
34	2,584	62	4,712	3	228	1	76			Norm. +	1.82	Negative	
32	2,560	64	5,120	2	160	2	160			Norm. +	1.63	Hæm. +	95
28	2,828	67	6,767	2	202	3	303			Nil	1.62	Hæm. +	55

TABLE XI

GROUP IV. *Animal No. 13.* *(Weight 11½ lbs.)*

M. & B. 693 dissolved in
0.06 g. per

Date.	Wt. in lbs.	Drug given.	Hæmoglobin %.	Hæmoglobin in g.	R.b.c. per cmm. in millions.	Reticulo %.	C.V.	M.C.V.	M.C.H.	M.C.H.C.	Total W.B.C. per cmm.
27.9.40	9.5	Blood	100	13.75	4.840	0.7	50.0	103.3	26.3	27.5	11,150
7.10.40		Blood	112	15.40	5.320	0.5	50.0	93.9	28.9	30.8	10,150
22.10.40		MB 693 in HCl. Blood before drug.	110	15.12	5.520	0.6	49.0	88.7	27.4	30.8	10,500
Mean	11.5		107.3	14.75	5.227	0.6	49.6	95.3	27.5	29.7	10,600
23.10.40		2nd *day*. MB 693 in HCl. by stomach tube.									
24.10.40		3rd day do.									
25.10.40		4th day do. Blood	90.0	12.37	4.070	1.2	40.0	98.2	30.4	30.9	9,450
26.10.40		5th day do.									
27.10.40		6th day do.									
28.10.40	11	7th day do. Blood	80.0	11.00	4.070	2.4	38.8	95.3	29.4	29.3	6,050

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dilute Hydrochloric Acid

kilo body weight

0.328 g. daily for 7 days given by stomach tube.

Neutrophils		Lymphocytes		Large Monos.		Eosinophils		Basophils		Abnormal Cells.	Arneth Count.	Van den bergh.	Coagulation time in secs.
%	Total	%	Total	%	Total	%	Total	%	Total				
33	3,679	60	6,690	3	335	4	446			Nil	1.29	Negative	
										Nil		,,	50
33	3,465	60	6,300	2	210	5	525			Nil	1.23	,,	50
											1.26	Negative.	50
55	5,197	39	3,686	1	94	5	473			Nil	1.56	Hæm. +	
37	2,238	56	3,388	2	121	5	303			Norm –	1.31	Hæm.	50

TABLE XII

GROUP V. *Animal No. 2 Control Monkey.*

Date.	Wt. in lbs.	Drug given.	Haemoglobin %.	Haemoglobin in g.	R.b.c. per cmm. in millions.	Reticulo %.	C.V.	M.C.V.	M.C.H.	M.C.H.C.	Hotal W.B.C. per cmm.
25.7.40	8.		92.	12.65	5.61	3.1	44.0	78.3	22.5	28.75	19,050
1.8.40	8.		94.0	12.92	5.57	1.1	43.0	77.1	23.2	30.0	15,600
8.8.40	8.		91.0	12.51	5.27	1.9	38.2	72.4	23.7	32.6	17,600
21.8.40	8.5		94.0	12.92	5.37	1.1	41.0	76.3	24.1	31.5	11,350
Mean	8.125		92.8	12.75	5.45	1.8	41.5	76.03	23.375	30.712	15,900
21.9.40	9.		108.0	14.85	6.06	0.7	48.0	79.2	24.5	30.9	13,500
14.10.40	9.		104.0	14.30	6.02	0.6	46.0	76.4	23.7	31.1	17,900
21.10.40	9.		110.0	15.12	5.62	0.6	45.0	80.7	26.9	33.6	12,300

TABLE XIII

GROUP V. *Animal No. 6 Control Monkey.*

Date.	Wt. in lbs.	Drug given.	Haemoglobin %.	Haemoglobin in g.	R.b.c. per cmm. in millions.	Reticulo %.	C.V.	M.C.V.	M.C.H.	M.C.H.C.	Hotal W.B.C. per cmm.
30.7.40	7.5		92.0	12.65	5.33	0.8	38.0	71.3	23.7	35.3	16,800
5.8.40	8.		95.0	13.06	5.43	1.4	40.0	73.7	24.0	32.5	14,900
9.8.40	8.		94.0	12.92	5.36	0.8	46.0	85.8	24.1	28.1	14,800
22.8.40	8.5		93.0	12.78	5.34	1.0	38.0	71.1	23.9	33.7	16,550
Average Mean	8.		93.5	12.85	5.36	1.0	40.5	75.47	23.9	31.9	15,762
21.9.40	8.5		99.0	13.61	5.64	1.2	43.0	76.2	24.1	31.6	14,950
4.10.40	9.0		110.0	15.12	5.87	1.0	44.0	74.9	25.7	34.3	15,250
Average mean of 6 examinations.			97.16	13.36	5.49	1.0	41.5	75.5	24.25	32.25	15,540

Neutrophils %	Neutrophils Total	Lymphocytes %	Lymphocytes Total	Large Monos %	Large Monos Total	Eosinophils %	Eosinophils Total	Basophils %	Basophils Total	Abnormal Cells	Arneth Count	Van den bergh	Coagulation time in secs
50	9,525	45	8,872	4	762	1	191			Nil	1.75	Negative	45
30	4,680	64	9,984	5	780	1	156			,,	1.67	,,	40
												,,	35
40	4,541	57	6,740	1	113	2	226			,,	1.46	,,	40
40	6,248	55	8,432	3.3	551	0.96	190			Nil	1.57	Negative	40
32	4,320	60	8,100	3.0	405	5	675			Nil	1.42	Negative	
36	6,444	60	10,740	2.0	358	2	358			,,	1.38	Hæm.+	
32	3,936	62	7,626	2.0	246	4	492			,,	1.47	-Ve.	45

Neutrophils %	Neutrophils Total	Lymphocytes %	Lymphocytes Total	Large Monos %	Large Monos Total	Eosinophils %	Eosinophils Total	Basophils %	Basophils Total	Abnormal Cells	Arneth Count	Van den bergh	Coagulation time in secs
48	8,064	40	6,720	9	151.2	3	504			Nil	1.64	Negative	
45	6,705	50	7,450	3	373	2	372			,,	1.65	,,	
												,,	
35	5,792	63	10,427	2	331					,,	1.51	,,	45
42.6	6,853	51	8,199	4.6	738.6	1.6	292			Nil	1.60	Negative	45
20	2,990	74	11,063	4	598	2	299			Nil	1.85	Negative	40
29	4,422	67	10,218	2	305	2	305			,,	1.35	,,	40
										,,	1.60		42

FIG. I. Effects of Prontosil Soluble by Intramuscular Injections on Animal No. 1.
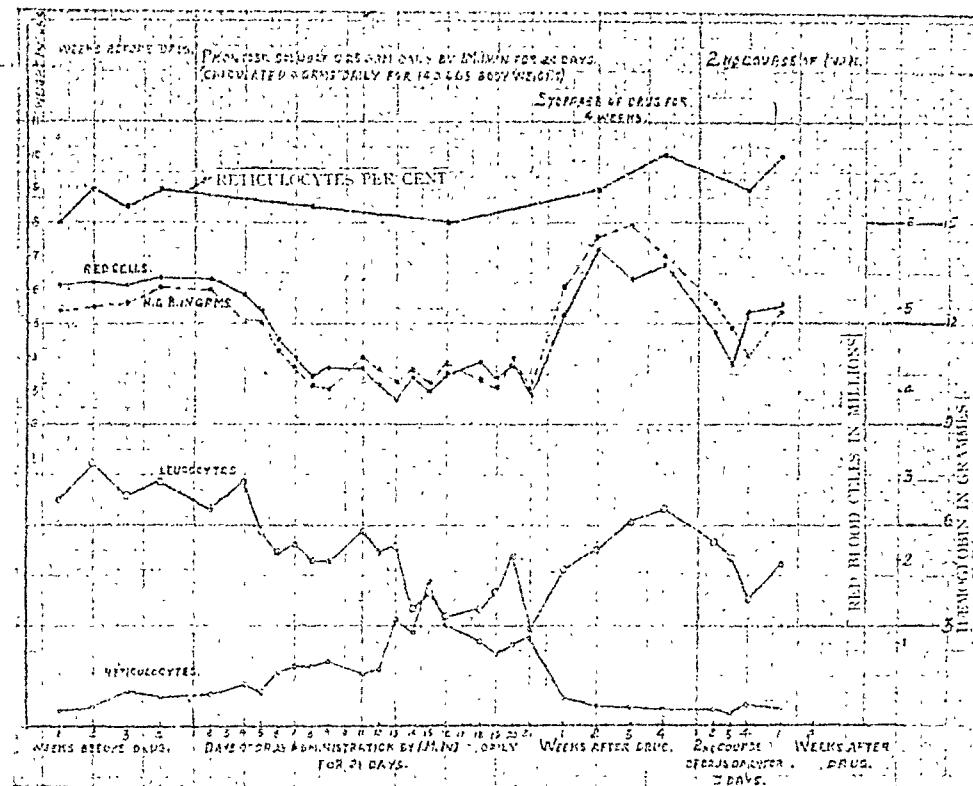
FIG. II. Effects of Prontosil Soluble by Intramuscular Injections on Animal No. 3.

Fig III Effects of Prontosil Soluble by Intramuscular Injections on Animal No 2.
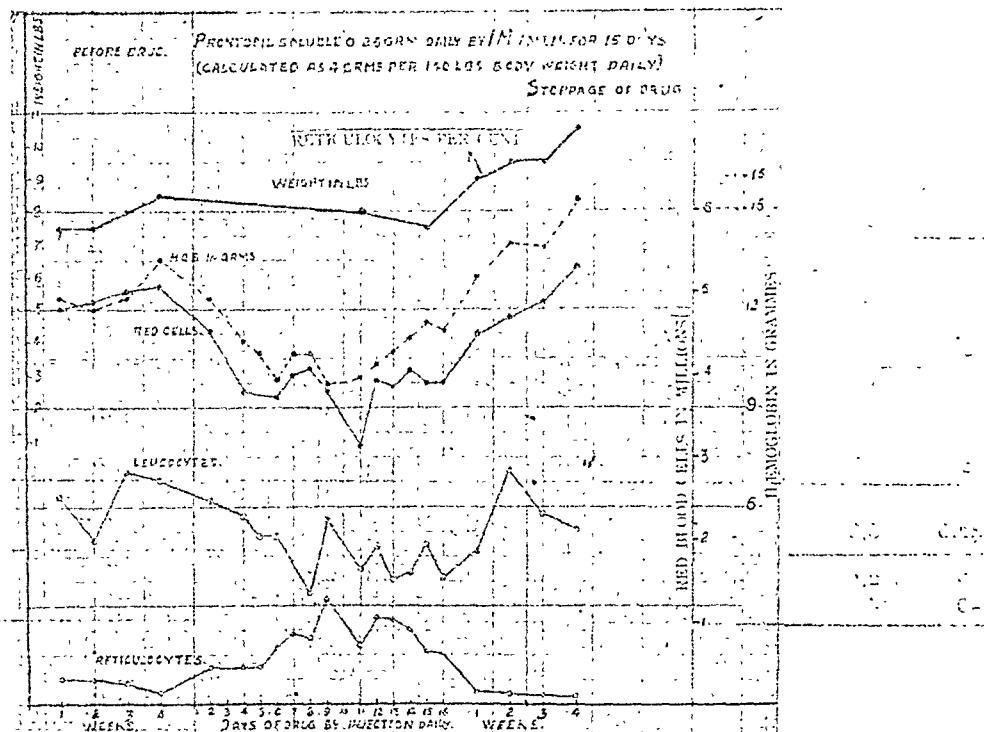
Fig IV Effects of Prontosil Album in Water Per Os on Animal No 5.
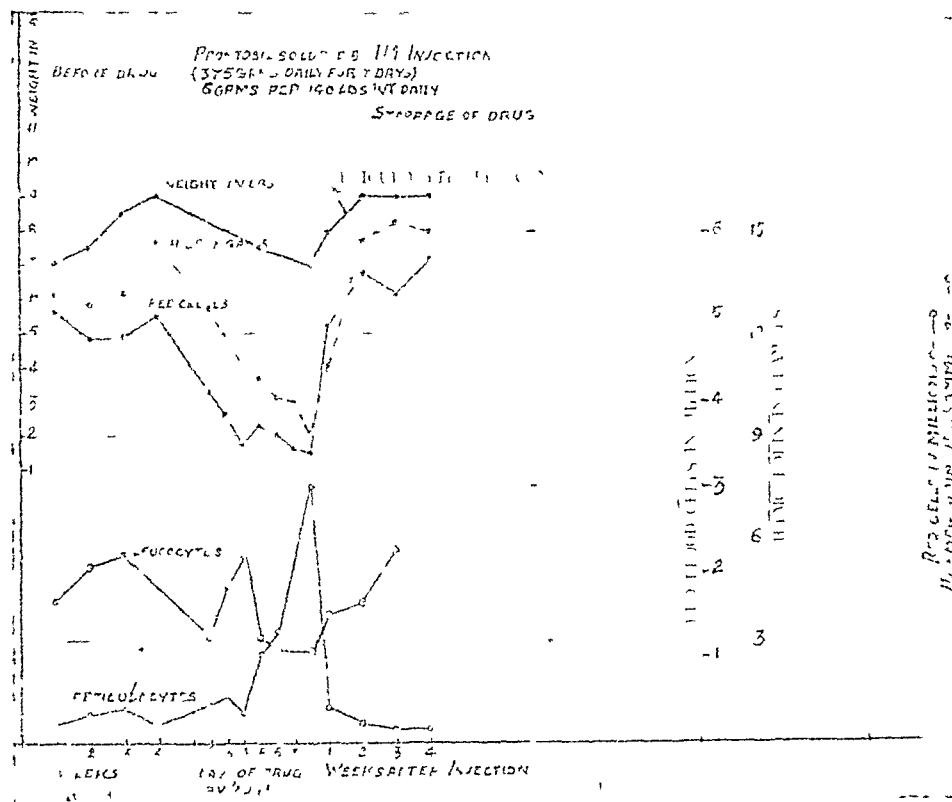
FIG. V. Effects of Prontosil Album in Water Per Os. on Animal No. 7.
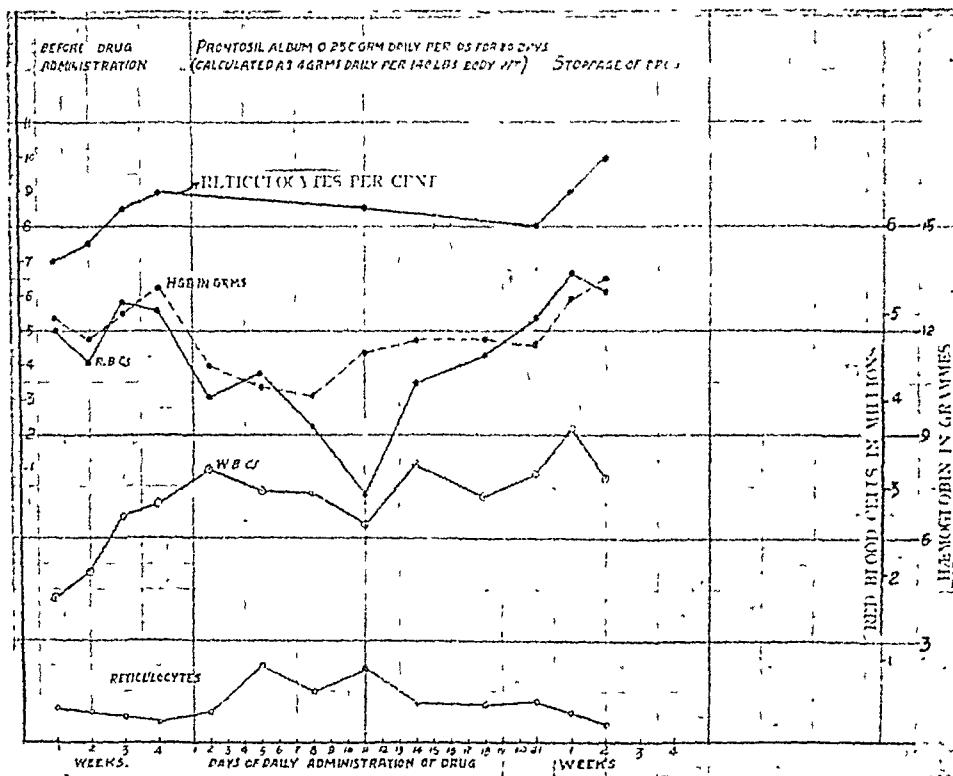
FIG. VI. Effects of Prontosil Album in Water Per Os. on Animal No. 8.
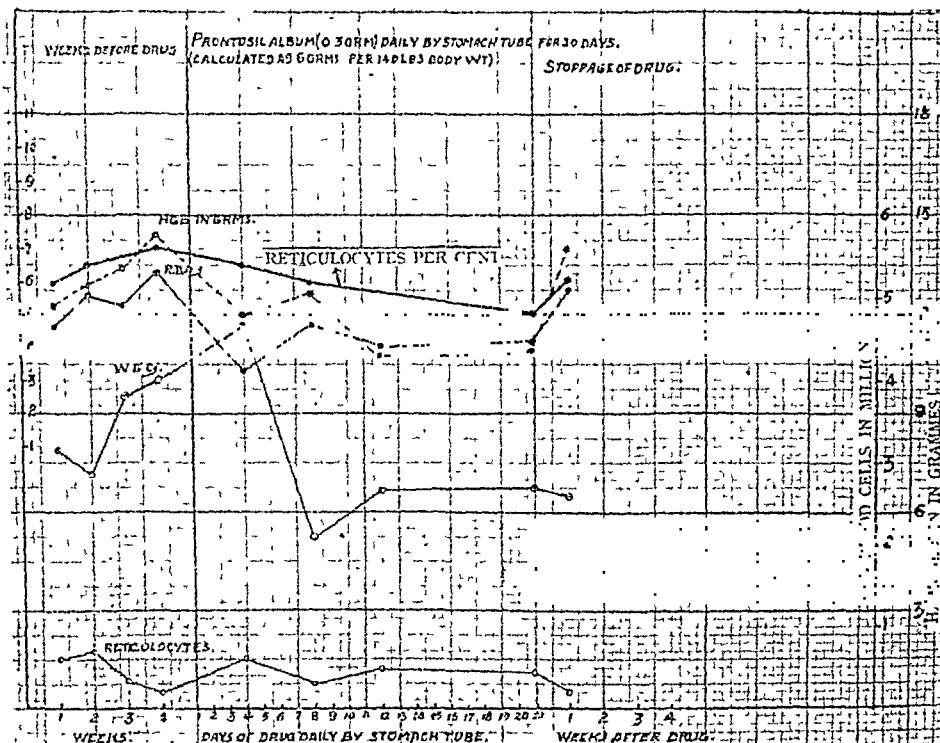
Fig. VII.   Effects of M & B 693 in Water Per Os. on Animal No. 10.
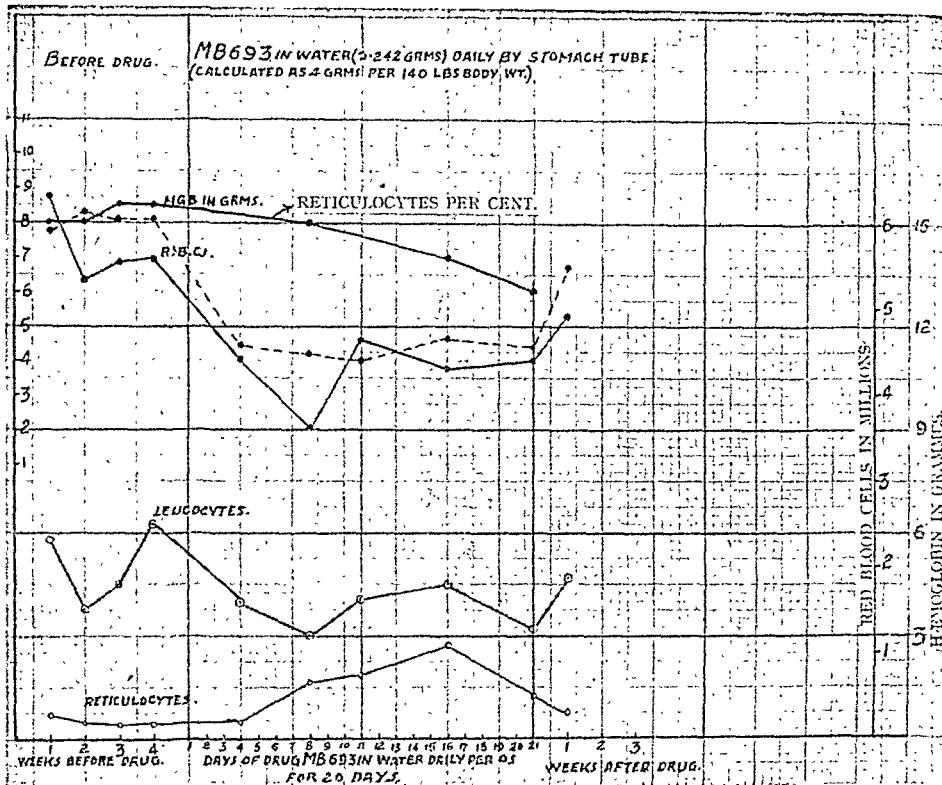
Fig. VIII.   Effects of M & B 693 in Water Per Os. on Animal No. 12.
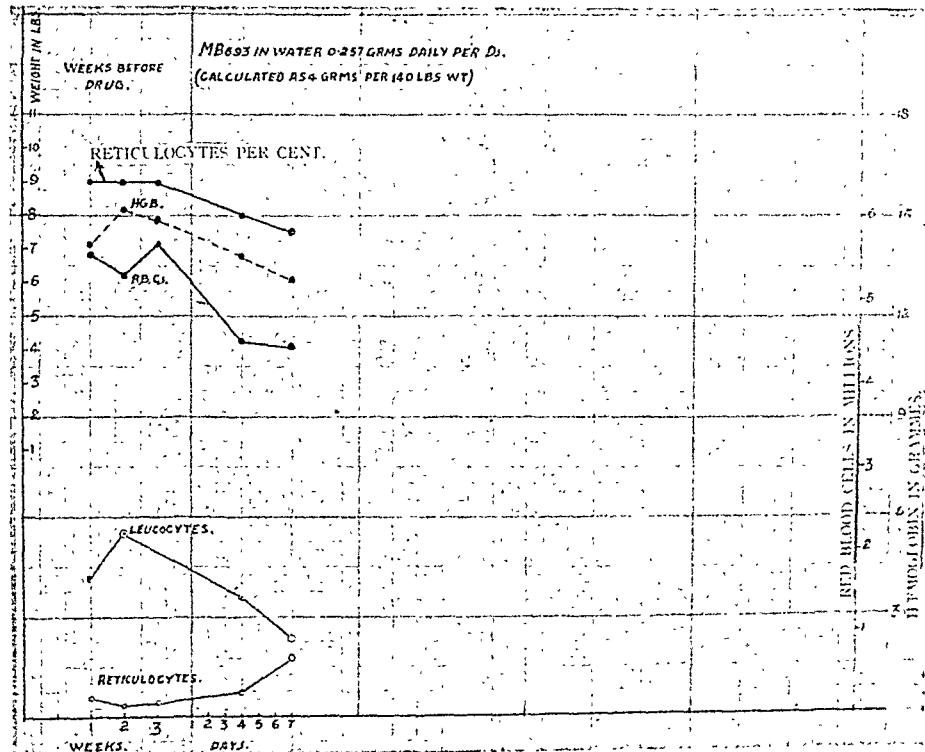
FIG. XI. Blood Examination Findings of Control Animals No. 2 and 6.
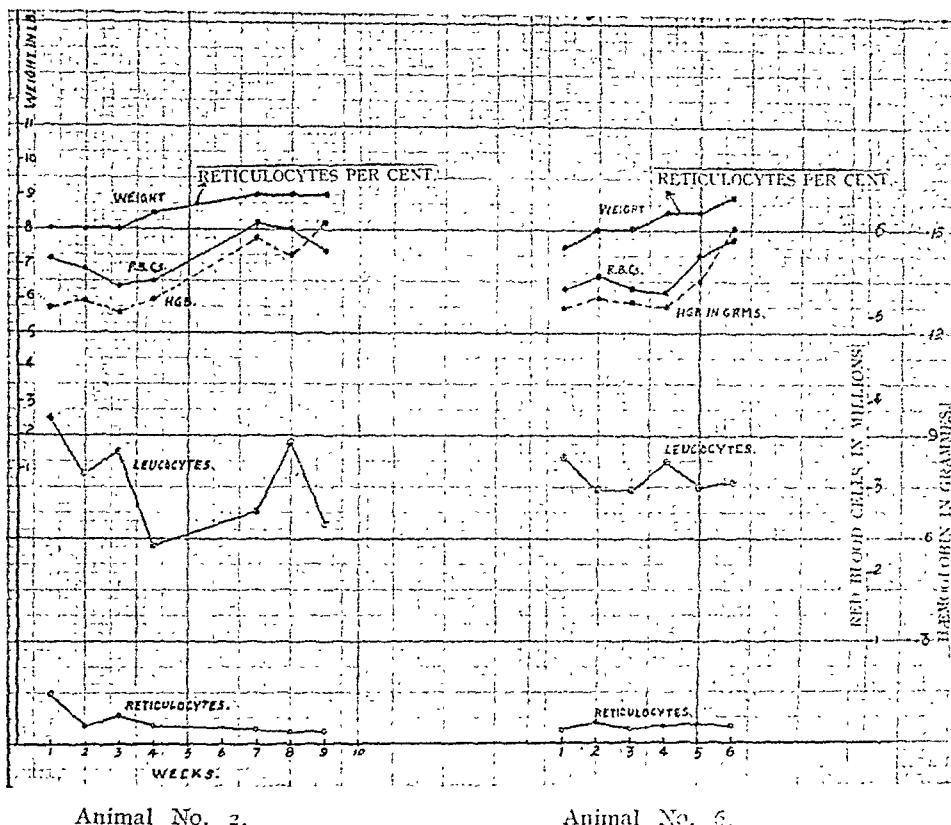
Animal No. 2.   Animal No. 6.

FIG. XII. Blood Concentration of Prontosil Album Per Os. in Animal No. 8.
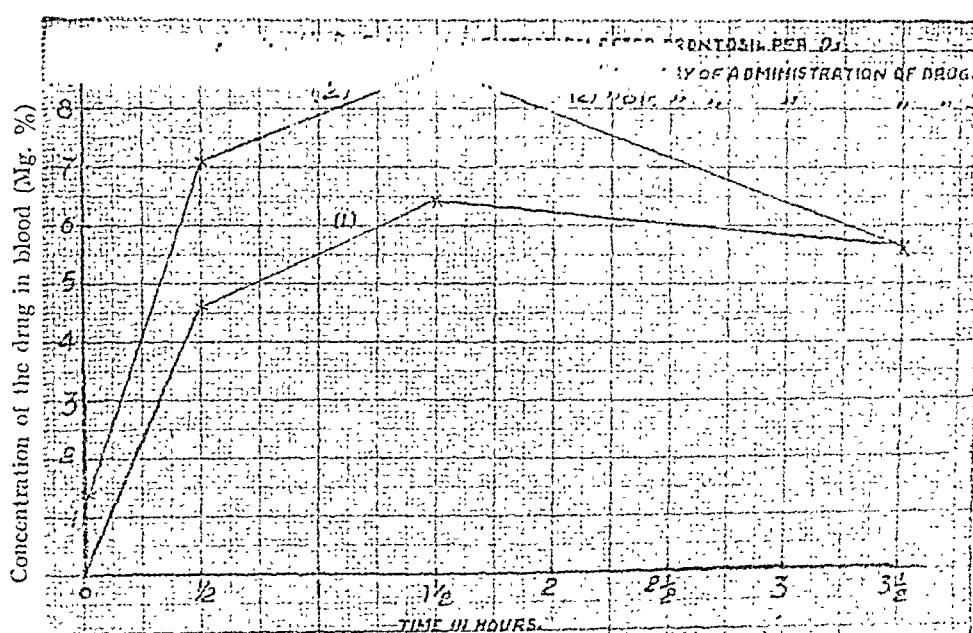
FIG. XV. Blood Concentration of M & B in Water Per Os. in Animal No. 12.

BLOOD CONCENTRATION FOLLOWING THE ADMINISTRATION
OF MB 693 IN WATER

(Concentration of the drug in blood (Mg. %))

(2)

(1)

0   ½   1   1½   2   2½   3   3½   4
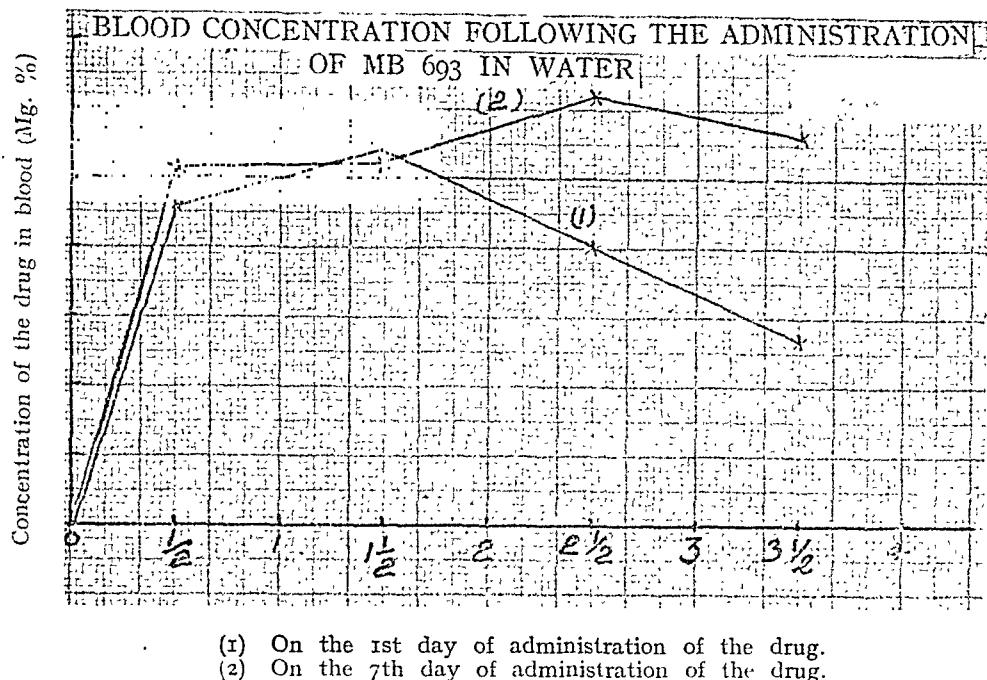
(1) On the 1st day of administration of the drug.
(2) On the 7th day of administration of the drug.

FIG. XVI. Blood Concentration of M & B 693 in Hcl Per Os. in Animal No. 11.

(Concentration of the drug in blood (Mg. %))

(1)

0   ½   1½   2   2½   3   3½

Time in Hours.
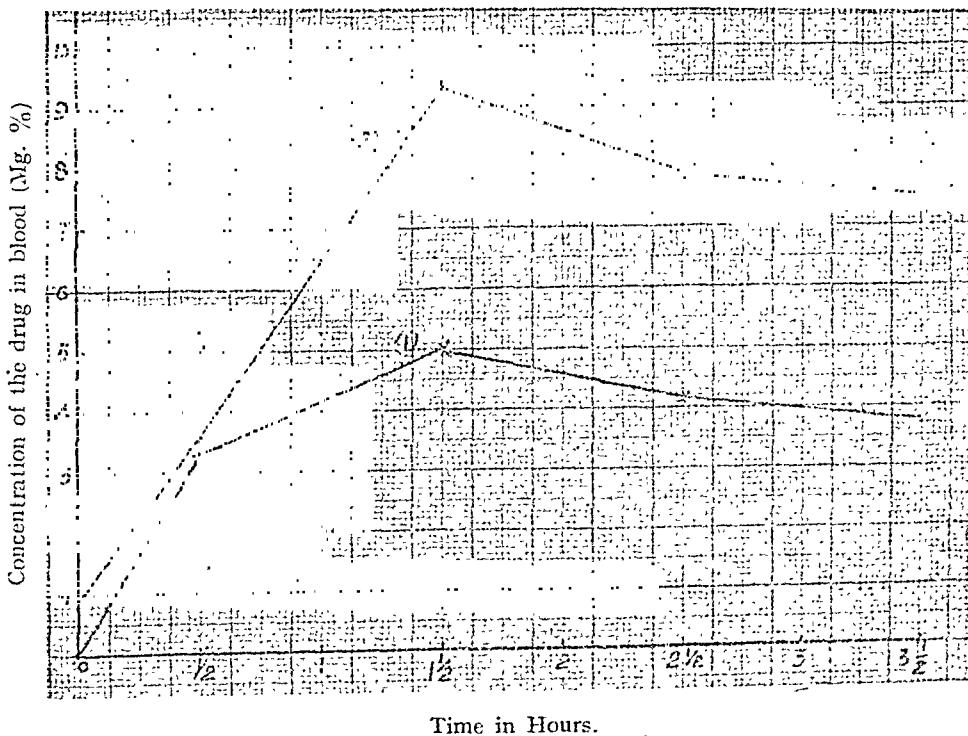
(1) After the 1st day of administration of the drug.
(2) After the 15th day of administration of the drug.

FIG. XVII. Blood Concentration of M & B 693 in Hcl Per Os. in Animal No. 13.
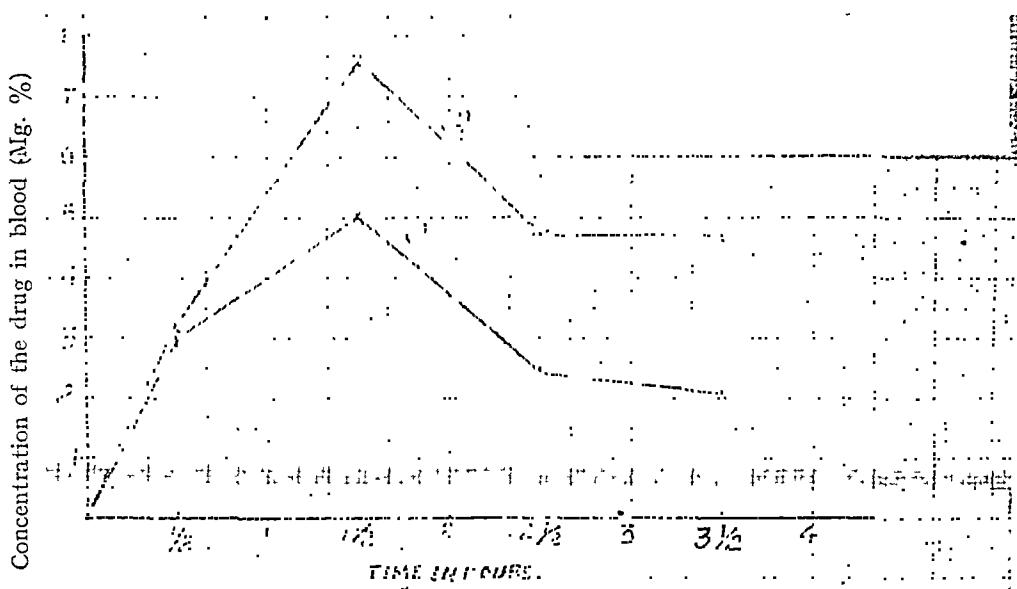
(1) On 1st 7th day of administration of the drug.
(2) On the 7th day of administration of the drug.

FIG. XVIII. Blood Concentration of Prontosil Soluble by Injection in Animal No. 4.
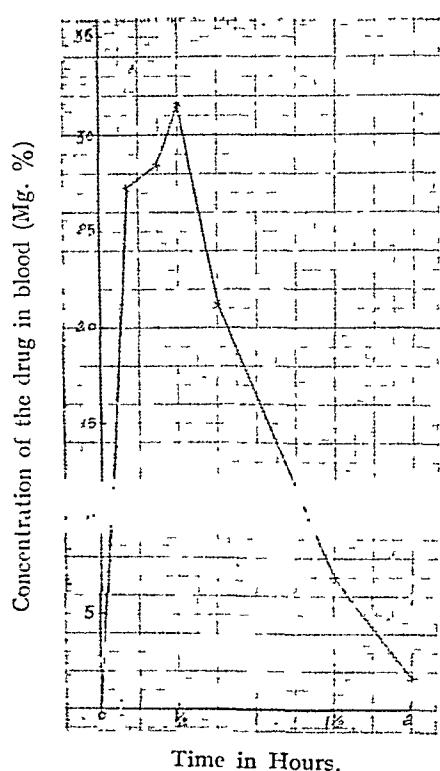
Time in Hours.

STUDIES IN VITAMIN A METABOLISM. PART II. ABSORPTION
OF MINIMAL DOSES OF β-CAROTENE BY VITAMIN A-
DEFICIENT RATS[*]

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According to the international standard, β-carotene has a potency of 1.67×10^6 I.U. per gram; vitamin A would have almost the same potency because of the widely accepted assumption that one molecule of β-carotene gives two of vitamin A by symmetrical hydrolytic fission.

But recent biological assays (1, 2) employing the crystalline vitamin and its esters show that the potency of pure vitamin A is in the region of 3.2×10^6 I.U. per gram ; in other words, 1 part of vitamin A is biologically equivalent to 2 parts of β-carotene. This discrepancy may be due to (*a*) incomplete absorption of the standard β-carotene, as contrasted with that of vitamin A, by the experimental animals, (*b*) incomplete conversion of the carotene into vitamin A *in vivo,* or (*c*) both these causes.

Of late, there has been some speculation on the possible manner of conversion of the provitamin into vitamin A (2-4). It has been suggested that the theory and the experimental findings could be reconciled if we assume an unsymmetrical fission, *i.e.,* alternately on either side of the 15-15′ carbon linkage, as the first step in the conversion. Then, a molecule of β-carotene can give rise to only one molecule of vitamin A and not two as has been hitherto believed.

While there is no direct experimental evidence in support of the above theory, there are some reasons to believe that the discrepancies may be due to incomplete absorption of the standard β-carotene. To state briefly, they are as follows: (*a*) It has been reported that experimental animals as also

human beings usually retain only 50 to 75% of the ingested carotene, the rest being excreted into the fæces (5-8). (b) There is a tacit assumption that all the β-carotene administered in the biological assay is absorbed by the experimental animals, but this has not been experimentally demonstrated. (c) De (7) reported an excretion of nearly 40% when a subminimal dose of 1 μg. of carotene was administered per rat per day. This experiment was, however, conducted on adult rats which were not depleted of their vitamin A reserves ; carotene of unknown composition was employed instead of β-carotene and the identity of the fæcal carotene was not established.

It was therefore thought to be of interest to study the extent to which β-carotene administered at test dose levels and under the experimental conditions of the biological assay can be absorbed by the rats.

EXPERIMENTAL

The experimental details with regard to the preparation of the test animals (albino rats), administration of the supplement and estimation of carotene in the fæces were the same as those described in the preceding paper (9). Crystalline β-carotene obtained from the S.M.A. Corporation was dissolved in arachis oil and used for the supplement. The fæces were collected on alternate days and preserved in the refrigerator. In order to get estimable quantities of carotene, it was necessary to collect the fæces of each group of animals for 10 to 14 days before analysis could be performed.

In a preliminary experiment, the absorption of carotene was studied at three levels of intake, viz., 1, 2 and 4 micrograms per rat per day[1], and the results are presented in Table I. The higher values for absorption with increasing levels of intake are exactly the opposite of what is normally to be expected. It was thought that the reason for this might be found in the possibility of a part of the pigment being of a non-carotene nature (8). Therefore, the fæcal "carotene" solution and the pure β-carotene solution were examined in a visual spectro-photometer.[2] The absorption curves A and B in Fig. 1 indicate the presence of some non-carotene pigment in the fæcal "carotene" solution. Further confirmation was obtained when analysis of the fæces of rats maintained for sometime on the vitamin A-free basal diet gave an apparent carotene excretion value of 0.2-0.4 μg. per rat per day. On spectroscopic examination, however, the solution showed no absorption maxima ; only a straight line graph (resembling Curve C in Fig. 1) was obtained.

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1. Six days in a week.
2. The authors' thanks are due to Dr. J. C. Ghosh for permission to make use of the Gaertner Visual Spectrophotometer Assembly L489A set up in the Department of Pure and Applied Chemistry of this Institute. The help given by Dr. S. K. Bhattacharya in this connection is thankfully acknowledged.
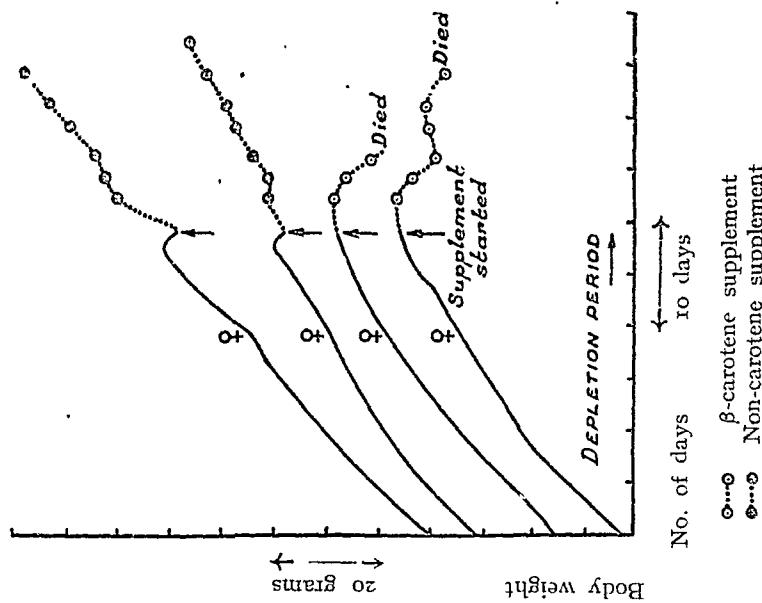
FIG. 2. Biological Assay of the excreted pigments.
Level of dosage: 2 μg per rat per day.

○···○ β-carotene supplement
◉···◉ Non-carotene supplement
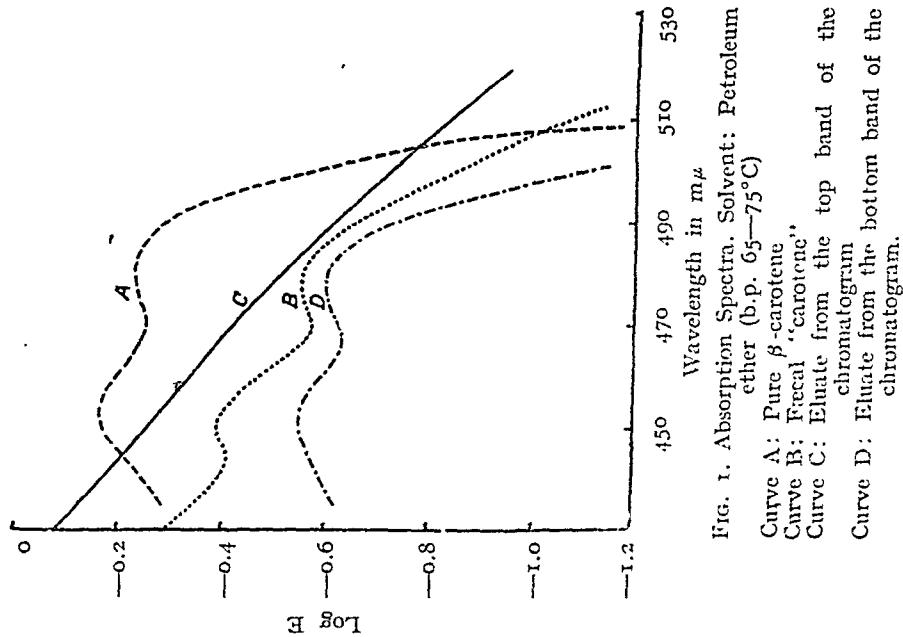
FIG. 1. Absorption Spectra. Solvent: Petroleum ether (b.p. 65—75°C).

Curve A: Pure β-carotene
Curve B: Fecal "carotene"
Curve C: Eluate from the top band of the chromatogram
Curve D: Eluate from the bottom band of the chromatogram.

Chromatographic Analysis.—In the next experiment in which absorption was studied at 1 and 2 μg. levels, an attempt was made to separate the β-carotene from the non-carotene impurities by subjecting the fæcal "carotene" extracts to chromatographic analysis. The following procedure was adopted.

An adsorption tube of 1.8 cm. diameter was packed uniformly with Brockmann's alumina (Merck) to a height of 12 cm. The concentrated epiphasic extract of the fæcal pigments (about 5 cc.) was drawn through the absorbent column and the chromatogram was developed with petroleum ether (b.p. 60-75°) under mild suction. A pale yellow upper band and a lower pink band were obtained. These were eluted separately with petroleum ether (b.p. 60-75°) containing 5% absolute alcohol. The elutes were made up to suitable volumes and examined in the spectrophotometer. The absorption spectra of these solutions (Fig. 1, Curves C and D) show clearly that the bottom pink band is that of β-carotene and the top band that of the non-carotene impurity. Thus, during the five week experimental period, the fæces of each group of rats were analysed thrice and the true carotene content determined in each case after chromatographic separation. The same procedure was followed in all subsequent experiments described in this paper. The purity of the carotene solutions was tested in every case by spectrophotometric examination ; but only values obtained by the colorimetric comparision are presented here.

The results of this experiment (see Table II) show that considerable amounts of carotene were excreted by the animals even at these very low levels of dosage. It will be observed, however, that the recoveries of the pigments on chromatography were never quantitative ; they varied from 40 to 66% of the original. Experiments were therefore conducted to determine the recovery of carotene when mixtures of known composition were analysed similarly. The non-carotene pigment was prepared from the fæces of animals subsisting on a carotenoid-free diet. Aliquots of this extract were added to known amounts of β-carotene in solution and after concentration to low volumes, the mixtures were chromatographed on columns of Brockmann's alumina in the manner described above. The separated pigments were estimated and the recoveries obtained in each case are presented in Table III. An examination of these figures shows that (*a*) the recovery of carotene is high while that of the non-carotene pigment is very poor and (*b*) an increase in the amount of non-carotene pigment adversely affects the recovery of β-carotene. However, it can be safely assumed that, in the earlier experiment (*vide* Table II) about 90% of the β-carotene should have been recovered on chromatographic analysis. Calculating on this basis, the excretions of β-carotene at the levels of 1 and 2 μg. per rat per day would work out to be 14.2 and 12.3% respectively.[3]

3. Similar values have been obtained by Sherman (10) in an experiment on rats extending over 7 days, daily supplement being 5 μ g. of β-carotene.

Effect of increasing the dietary fat.—It is a well known fact that the absorption of carotene is very poor when the fat content of the diet is low. Highest absorptions are obtained when the diet contains adequate amounts of fat and when carotene is administered through the medium of a suitable oil. The basal diets used in the experiments so far described, contained 3% of coconut oil. This may be quite adequate for the optimum absorption of carotene but since as much as 10% of oil is being used in certain laboratories, further experiments were conducted employing a basal diet containing 10% coconut oil. The results are given in Table IV. Assuming the carotene recovery on chromatographic analysis to be 90%, the excretions account for 12.4 and 12.0% of the carotene administered at the levels of 1 and 2 μg respectively. These values are not significantly lower than those obtained in the previous experiment showing thereby that the increased intake of oil did not result in any appreciable improvement in absorption.

The animals of the above experiment were continued for another fortnight on the same basal diet and supplements but employing a freshly expressed sample of linseed oil as the medium for β-carotene. During that period 96 μg. and 168 μg. of β-carotene were ingested and the excretions were 12.2 μg. (13.7%) and 46.5 μg. (27.6%) respectively. The absorption in the first group did not alter materially but it was poorer in the other group, where a trend towards decreased absorption was noticed during the earlier periods (*vide* Table IV, 2 μg. level). Based on this single protocol, it is *not possible to conclude that the linseed oil has had an adverse effect on* absorption but it is of interest to note that no improvement was produced either.

Biological assay of the fæcal carotene.—Though the spectroscopic data may suffice to establish the identity of the fæcal carotene, it was considered desirable to confirm the same by growth experiments also. With this idea in view, the pigment fractions separated in the course of this investigation were collected and preserved in brown-glass bottles in the refrigerator. After evaporation of the solvent they were taken up in arachis oil and fed to vitamin A-deficient rats to correspond to a daily intake of 2 μg. per rat. On account of the small quantities of the pigments available, it was not possible to include more than two rats in each group for this experiment. But the results, represented graphically in Fig. 2, leave no doubt as to the identity of the β-carotene and the non-carotene pigment fractions obtained from the fæces.

It should be mentioned in this connection that the fæcal extracts were found to contain large amounts of sterols, part of which came down as bulky precipitates and caused considerable difficulty in washing the extracts free from alcohol. This precipitate which was separated by filtration, was

8

brownish yellow in colour. It was further observed that, on chromatographic analysis, the top band fractions invariably contained most of the sterols. The non-carotene pigment is evidently associated with these sterols.

DISCUSSION

These experiments demonstrate the fact that even at test dose levels, β-carotene is not completely absorbed by vitamin A-deficient rats. In the case of animals receiving 1 or 2μg. of β-carotene per day, it was possible to recover about 10 to 15% of it from their fæces by the available methods of analysis. In view of the unavoidable delays involved, it is probable that an appreciable portion of the excreted carotene had undergone destruction before the analysis could be performed. Further, the possibility of a part of the ingested carotene being destroyed in the physiological system itself cannot be ignored. It has been reported (7, 8) that, after a single dose of carotene is fed, rats continue to excrete it in their fæces for three or four days ; in the case of human beings it has been observed (11) to extend up to seven days even. During such a long stay in the digestive and intestinal tracts, it is certain that a partial destruction of carotene does take place. But, unfortunately, these losses cannot be easily estimated nor can they be altogether avoided. Accounting for all these factors, it would appear that the actual excretions might be higher than what these figures would indicate.

Vitamin A, on the other hand, has been shown to be absorbed more quickly (12, 13) and almost quantitatively at far higher levels of intake (6, 7, 11). About half an hour after ingestion of a liver oil the presence of vitamin A can be detected in the livers of previously depleted rats while the storage to a large extent takes place within 6 hours. The transformation of β-carotene into vitamin A in the liver was observed to begin only two hours after the ingestion of carotene. Under varying conditions of feeding, absorption of vitamin A by adult rats was found by De (*loc. cit.*) to be almost complete while Wilson *et al* (6) could not detect any excretion when a highly concentrated extract of vitamin A was fed to an adult human subject. The work of Greaves and Schmidt (14) and of Drummond and co-workers (15) on the manner of absorption of vitamin A and carotene also supports the view that vitamin A is absorbed quantitatively and far too quickly for any appreciable destruction to occur in the physiological system.[4]

4. This refers only to levels approximating to the normal physiological requirements. Baumann *et al* (13) observed that only a small part of the ingested vitamin A could be recovered from the liver and in the absence of any faecal excretion they concluded that the major part of the vitamin A was destroyed in the digestive tract. Recent work of Moore (16) and Bacharach (17), however, has shown that a high intake of vitamin E is necessary for the optimum storage of vitamin A in the liver. The observation of Baumann *et al*, therefore, does not contradict the efficient utilisation of small amounts of vitamin A for normal growth and reproduction.

It is therefore suggested that partial excretion of the standard β-carotene might be the major factor responsible for the observed discrepancies.[5] A similar conclusion was arrived at by Wald *et al* whose note (11) reached the authors during the final stages of this investigation. They reported that carotene and xanthophyll (about 10 mg.) fed in cottonseed oil to adult human subjects were excreted to the extent of 60 and 8% respectively. But the excretion of vitamin A was found to be negligible when the daily intake was less than 25,000 I.U.

The present findings further emphasise it as a physiological property characteristic of these pro-vitamins that they cannot be completely absorbed by the animal system even under the most favourable conditions. It is, therefore, no matter for surprise that the absorption of carotene was found to vary inversely as the level of intake. In the case of vitamin A, however, a threshold value should be reached before excretion could be detected and a similar relation could be observed (11).

SUMMARY

Absorption of minimal doses of β-carotene by vitamin A-deficient rats has been studied and it was observed that even under the conditions of the biological assay, considerable amounts of carotene were excreted by the experimental animals.

Analyses of the fæces of rats maintained on a carotenoid-free diet, gave an apparent carotene excretion value of 0.2-0.4μg. per rat per day ; spectroscopic examination showed this to be due to a non-carotene impurity possessing only a general absorption.

By employing the chromatographic technique, the carotene could be separated from the associated pigments and estimated quantitatively. It was possible to recover from the fæces, about 10 to 15% of the β-carotene administered at the levels of 1 and 2μg. per rat per day. Increasing the dietary fat to 10% did not materially improve the absorption. The identity of the fæcal β-carotene and non-carotene pigment was established by growth experiments on rats.

The recent hypothesis suggesting that the pro-vitamins undergo an unsymmetrical fission *in vivo* has been discussed in the light of these findings.

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5. Another possible factor is suggested by the finding of Wilkinson (*Biochem. J.* 1941, **35**, 824) that the sample of β-carotene isolated from palm oil by Hunter and Scott (*Ibid*, 1941, **35**, 31) possessed a potency of 2×10^6 I.U. per gram. The natural conclusion "that the β-Carotene forming the International Standard was only 90% pure was confirmed by spectrophotometric data by him."

Results essentially similar to those presented in this paper have been obtained in another experiment (2μg. level) where coconut oil (Tomco's) was used as the medium for β-carotene. Nearly 700μg. of β-carotene were ingested and 60μg. were recovered from the fæces. Calculating on the basis of 90% chromatographic recovery, the excretion accounts for about 10% of the ingested carotene. In this respect, therefore, coconut oil is not far superior to arachis oil.

TABLE I

Absorption of Minimal Doses of β-Carotene by Vitamin A-deficient Rats.

Two male rats were used in each group. Experimental period was 8 weeks. All the animals survived and resumed growth. Basal diet consisted of extracted casein 15%, dried yeast 8%, salt mixture 5%, Tomco's coconut oil 3% and rice flour 69% ; calciferol supplement 0.35μg. per rat per week.

β-Carotene (μ g.) administered per rat per day	1	2	4
Total amount of β-carotene (μ g.) administered to the group	96	192	384
Total "carotene" (μ g.) excreted	48.7	56.3	78.0
Apparent absorption (per cent.)	49	70.7	79.7

TABLE II

Absorption of Minimal Doses of β-Carotene by Vitamin A-deficient Rats.

Each group consisted of 4 male and 3 female rats. All the animals survived the five-week experimental period and resumed growth. Basal diet was the same as for the preliminary experiment. The figures represent micrograms of β-carotene or equivalent of non-carotene pigment.

No. of days	One microgram level				Two microgram level			
	Amount ingested	Apparent excretion	On separation		Amount ingested	Apparent excretion	On separation	
			Non-carotene	β-carotene			Non-carotene	β-carotene
10	59.4	29.4	6.3	5.9	118.7	40.5	9.2	9.3
14	86.1	43.5	10.2	12.9	172.2	58.5	13.9	18.0
13	79.0	42.0	10.8	9.9	158.0	60.0	17.3	22.5
Total 37	224.5	114.9	27.3	28.7	448.9	159.0	40.4	49.8

Average growth response = 37 g.   Average growth response = 44 g.

TABLE III

Recoveries of β-Carotene and the Non-carotene pigment on Chromatographic Analysis of their Mixtures.

The values are expressed in micrograms of β-carotene or equivalent of non-carotene pigment.

Experiment No.	Composition of the mixture		Recoveries		
	β-carotene	Non-carotene	Total	β-carotene	Non-carotene
1	21.6	20.0	66%	96%	36%
2	43.2	20.0	72%	92%	43%
3	15.0	30.0	49%	85%	32%

TABLE IV

Absorption of Minimal Doses of β-Carotene by Vitamin A-deficient Rats.

Basal diet consisted of extracted casein 18%, salt mixture 5%, coconut oil 10%, dried yeast 8%, and rice flour 59% ; Calciferol supplement 0.35 μg. per rat per week. The experimental period was 6 weeks. The 1 μ g. group consisted of 4 male and 2 female rats and the 2 μg. group of 4 male and 3 female rats. All the animals of the latter group survived and resumed growth. One male and one female rat of the 1 μg. group died on the 11th and 20th day respectively. Two female vitamin A-deficient rats were added to this group for the last week of the experiment. The following figures represent micrograms of β-carotene or equivalent of non-carotene pigment.

No. of days	One microgram level				Two microgram level			
	Amount ingested	Apparent excretion	On separation		Amount ingested	Apparent excretion	On separation	
			Non-carotene	β-carotene			Non-carotene	β-carotene
16	87.0	55.5	18.8	9.6	208.0	63.0	22.5	17.2
14	54.0	19.5	5.9	3.6		49.5	17.2	10.8
14	63.0	42.0	11.3	9.6	8.0	78.0	19.7	31.5
Total 44	204.0	117.0	36.0	22.8	544.0	190.5	59.4	59.5

Average growth response (of 4 rats) = 38 g. Average growth response = 47 g.

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VITAMIN B₁ STUDIES. PART II. VITAMIN B₁ CONTENT OF SOME INDIAN FOODSTUFFS BY THE THIOCHROME METHOD USING A SENSITIVE PHOTOELECTRIC FLUORIMETER

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Mc. Carrison and Norris (1) and Mc. Carrison (2) determined the relative vitamin B₁ values of Indian cereals by growth methods. Acton *et al* (3) estimated the vitamin B₁ content of samples of Indian rice by the method of Spruyt (4). Ghosh and Guha (5) and Wilson *et al* (6) studied the vitamin B₁ content of Indian foodstuffs by biological methods. Recently, Passmore and Sundararajan (7) used the thiochrome method for the study of vitamin B₁ content of millets according to the method of Aykroyd *et al* (8). The present investigation deals with the estimation (thiochrome method) of vitamin B₁ content in cereals, millets and pulses, according to the quick and simple procedure (Method I) developed by Murty and Rau (9).

EXPERIMENTAL

Material.—The paddy samples were sun-dried for a definite time, dehusked in a wooden huller (with practically no loss of bran), powdered and preserved in glass-stoppered bottles for analysis. The other cereals, millets

and pulses (with husks) were powdered and representative samples taken for vitamin B_1 estimation.

Procedure.—To three glass-stoppered, narrow-mouthed bottles (200 cc. capacity), 5 g. of the powdered sample of the material under investigation are weighed out accurately in each. To the second and third bottles 1 and 2 cc. respectively of standard stock vitamin B_1 solution are pipetted out, in order to estimate (in duplicates) the percentage of recovery of added vitamin B_1. Then 46.5, 45.5 and 44.5 cc. of 0.5 N-hydrochloric acid are added to the first, second and third bottles respectively, to make up the total volume in each case to 50 cc. All the bottles are tightly stoppered and' shaken for one hour on a shaking machine. Then the contents are centrifuged for about 5 minutes. Three cc. of the centrifugate from each are taken for the estimation of observed value, and percentage of recovery (in duplicates) of added vitamin B_1, respectively, according to the original method of Jansen (10). For 3 cc. of the extract it is found in general that 1 to 1.5 cc. of 1% potassium ferricyanide is the optimum quantity. For every set a "blank" is carried out without the addition of potassium ferricyanide. Two different standard vitamin B_1 solutions (converted to thiochrome) are tested every time a reading is taken to determine the sensitivity of the fluorimeter.

The "computed" vitamin B_1 value in all these estimations is calculated assuming that the percentage of recovery of estimated vitamin B_1 is the same as that of added vitamin B_1.

The vitamin B_1 content of at least one sample in each group of cereals, millets and pulses was estimated according to the procedure (Method II) of Pyke (11) as modified by Booth (12). In every case special care was taken to add enough alkali to the peptic digest (usually 3-4 cc. of N-sodium hydroxide) to give a pH of 4-5 (optimum for the activity of the phosphotase present in the taka-distase). Great care is taken to avoid formation of emulsions while extracting the thiochrome by *isobutyl* alcohol from the aqueous phase, specially in the cases of *Eleusine coracana* and *Sorghum vulgare*. The formation of the emulsion is easily avoided if shaking is not violent.

One cc. of ethyl alcohol is used for clearing up turbidity of the *isobutyl* alcohol layer, and 10 cc. of the clear *isobutyl* alcohol extract are used for fluorescence measurements in special experimental tubes.

Generally, the *isobutyl* alcohol extract is slightly tinged, the nature of which depends upon the colour of the acid extract of the material under investigation. This does not vitiate the results since suitable filters are used (see Murty and Rau, 9). There is no necessity to destroy the colour imparted to the *isobutyl* alcohol layer in these cases by the addition of hydrogen peroxide as recommended by McFarlane and Chapman (13). The results (average of at least three estimations in each set) are given below.

TABLE I

		Recovery of added vitamin B₁	Vitamin B₁ per 100 g.	
			Method I	Method II

1. *Cereals.*

(a) Rice (*Oryza sativa*).

Co. 9,	Coimbatore	75%	480γ	485γ
Adt. 11,	,,	85	360	—
Co. 4,	,,	80	400	—
G.E.B. 24,	,,	85	310	—
G.E.B. 24,	Mysore	70	310	—
G.E.B. 24,	Berhampur	80	250	—
Latisail (Amon),	Bengal	80	210	—
Kataktara (Aus),	,,	80	220	—
Boro Jagli,	,,	75	445	435
Nakanda,	Kangra	75	210	—
Gurumati,	C.P.	85	260	—
16 B.K.,	Bihar	84	260	—
Do.	Cuttack	85	320	—
Coimbatore Sanna,	Bangalore market	82	235	—

(b) *Triticum vulgare.*

Bansi	85	340	400
Sarabathi	90	420	500
Dharwar	70	420	420
Samba	80	250	280

Samples were purchased in the Bangalore market.

2. *Millets.*

(a) *Eleusine coracana.*

H. 22,	Bangalore	53	330	335
E.C. 593,	Coimbatore	54	400	—
E.C. 2985,	,,	60	300	—
E.C. 3517,	,,	53	370	—
E.C. 3735,	,,	60	270	280
E.C. 1540,	,,	80	700	—

(b) *Sorghum vulgare.*

As. 29,	Coimbatore	55	380	400
As. 2095,	,,	75	490	—

3. *Pulses.*

Bengal-gram. (*Cicer arietinum*, Linn)	80	380	470
Green-gram (*Phaseolous mungo*)	52	185	510
Horse-gram (*Dolichos biflorus*)	53	70	520
Black-gram (*Phaseolous radiatus*)	40	260	320
Field-bean@ (*Dolichos lablab*)	—	—	—
Dhal (*Cajanus indicus*)	67	270	725

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The results of Table I (3) suggest that the pulses investigated contain a good amount of their vitamin B_1 in some combined form.

The following table gives the vitamin B_1 content of rices dehusked·from the same variety of paddy harvested in different years.

TABLE II

Rice dehusked from paddy (Harvested in 1937-1938 and kept till 1941)		Rice dehusked from paddy harvested in 1941	
Variety.	Recovery of added vitamin B_1	Vitamin B_1 per 100 g. rice.	Recovery of added vitamin B_1	Vitamin B_1 per 100 g. rice.
Co. 9	70%	460γ	75%	480γ
Adt. 11	80	365	85 .	360
G.F.B. 24	85	300	85	310
Co. 4	80	370	80	400

The results of Table II show that the vitamin B_1 content of rice dehusked from the same variety of paddy harvested in 1937-38 and 1941 is the same.

DISCUSSION OF RESULTS

The results of Table I (1) show that raw dehusked varieties of Co. 9, Boro Jagli, Co. 4, and Adt 11 contain 400 to 500 γ vitamin B_1 while the rest 200 to 300γ vitamin B_1 per 100 g. Co. 9 raw husked rice contains the maximum vitamin B_1 content (480γ per 100 g.) among the several varieties investigated.

All the rices contain their vitamin B_1 in the free form. The percentage of recovery of added vitamin B_1 (Method I) is 70-85.

Four varieties of wheat were investigated for their vitamin B_1 content. Two of them (Bansi and Sarabathi) gave different values by the two methods suggesting that they contained a little combined vitamin B_1. This was confirmed by incubating the wheat overnight with $N/10$-HCl and estimating vitamin B_1 in the acid extract after digestion. The other two varieties (Table I, b) contain all their vitamin B_1 in the free form. This observation is in agreement with that of Booth (12) who reports that wheats contain all their vitamin B_1 in the free form. The percentage of recovery of added vitamin B_1 (Method I) is of the order of 70-90.

A number of coloured varieties of *Eleusine coracana* and one colourless variety (E.C. 1540) have been investigated for their vitamin B_1 content (Table I (2), a). It is found that E.C. 1540 (colourless) contains 700γ while the other coloured varieties about 300-400γ vitamin B_1 per 100 g. Another

point of interest is that the recovery of added vitamin B_1, (Method I) is 80% with E.C. 1540 while in the cases of coloured varieties it is about 50-60%. It is quite probable that the nature of the interfering substances (those that inhibit or quench the fluorescence of thiochrome) is associated more with the colouring matter. This aspect is being further investigated. *E. coracana* does not contain any combined vitamin B_1.

Sorghum vulgare contains about 400 γ vitamin B_1 per 100 g. and all of it is present in the free form.

All the pulses investigated (Table I, 3) contain combined vitamin B_1 to different extents. Horse-gram (*Dolichos biflorus*) contains very little (70 γ per 100 g.) as free vitamin B_1 while most of it exists in the combined form. *Cajanus indicus* contains the maximum (725 γ) while black-gram (*Phaseolus radiatus*) the least (320 γ) and the others excepting *Dolichos lablab* contain about 500 γ vitamin B_1 per 100 g.

The vitamin B_1 of a sample of field bean (*Dolichos lablab*) could not be estimated by either of the methods (I or II), since the blank (without potassium ferricyanide) and the experimental (with potassium ferricyanide) gave about the same reading. It appears therefore that the vitamin B_1 present in that field bean has already been oxidised by some enzyme or other agent. Experiments are in progress to find out whether the field bean contains preformed thiochrome. The confirmation or otherwise of the above observation will be reported in the near future.

The percentage of recovery of added vitamin B_1 (Method I) is very low especially in the case of *Phaseolus radiatus* which gives a very red solution when extracted with 0.5 *N*-acid. This again supports our view that the interfering substances are associated more with colouring matter.

Table II shows that the vitamin B_1 content of rice dehusked from paddy harvested at different times (1938 and 1941) is of the same order, suggesting that there is no loss of vitamin B_1. The slight difference either in the vitamin B_1 content or percentage of recovery of added vitamin B_1 in the rices, falls well within the experimental error.

SUMMARY

The vitamin B_1 content of a number of Indian food-stuffs has been estimated by the quick and simple procedure of Murty and Rau using a sensitive photo-electric fluorimeter.

It is found that all cereals (excepting two varieties of wheat) and millets contain their vitamin B_1 in the free form while the pulses contain it in the combined form in varying quantities.

The vitamin B_1 in one sample of *Dolichos lablab* examined seems to exist in the oxidised form (preformed thiochrome).

Our thanks are due to the following gentlemen who readily sent us the materials used in this investigation. Paddy and Millet Specialists to the Government of Madras, Economic Botanist to the Government of Bengal and Directors of Agriculture in C.P., Bihar, Orissa and Mysore. Our thanks are also due to Prof. V. Subrahmanyan for the interest evinced in the course of this investigation.

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STUDIES ON THE PROTEOLYTIC ENZYME OF WHITE GOURD (BENINCASA CRIAPRA)

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Investigations of fruits like papaya and pineapple have shown that they contain active proteases papain and bromelin with specific properties. Among the plant proteases are those described by Basu and Nath (1) obtained from *akanda* (*Calopropis Gigantia*) and by Chatterji, Ghosh and Chopra (2) from the seeds of *Butea frondosa*. In the present work, the common white gourd (*Benincasa Criapra*) was investigated regarding its protease.

EXPERIMENTAL

Preparation of the Proteolytic Enzyme

White gourd was minced and the juice, pressed out by means of a screw press, was cooled and treated with 90% alcohol. After centrifugation, the residue was dried in a vacuum desiccator. As shown later, the preparation had proteolytic activity. It was light grey in colour and the yield of the dry preparation was 3 to 4 g. per kilogram of white gourd used. It appeared to be fairly stable, if stored in a vacuum desiccator at 26° to 30°C. If properly prepared it retained its activity for over 3 months.

TABLE III

pH	No. of the flask.	Cc. of N/10 NaOH reqd. at the start.	Cc. of N/10 NaOH reqd. after incubation for 24 hours.	Hydrolysis after 24 hrs. (in cc. N/10 NaOH.)	Hydrolysis after 24 hrs. due to enzyme (in cc. N/10 NaOH.)
6.5	1.	7.2	11.9	4.7	4.7
	2.	3.0	3.0	0	
	3.	6.5	6.5	0	
7.0	1.	5.9	11.1	5.2	5.05
	2.	1.0	1.05	0.05	
	3.	5.4	5.5	0.1	
7.5	1.	5.2	11.0	5.8	5.45
	2.	0.8	1.0	0.2	
	3.	4.6	4.75	0.15	
8.0	1.	4.9	10.95	6.05	5.8
	2.	0.5	0.65	0.15	
	3.	4.1	4.2	0.1	
8.5	1.	3.5	9.9	6.4	6.1
	2.	0.2	0.3	0.1	
	3.	3.0	3.2	0.2	
9.0	1.	3.0	7.0	4.0	3.8
	2.	0	0	0	
	3.	2.5	2.7	0.2	

The above results show that optimum pH of the enzyme is 8.5 in the absence of any buffer.

Activity of the Enzyme at Different Temperatures

One g. of the dried enzyme preparation was made into a cream with water and the final volume was made up to 120 cc.

Flask No. 1 contained 10 cc. of 8% gelatin solution, 6 cc. of enzyme cream, 5 cc. borate buffer, and 0.5 cc. toluene.

Flask No. 2 contained 10 cc. water, 6 cc. of the enzyme cream, 5 cc. borate buffer and 0.5 cc. toluene.

Flask No. 3 contained 10 cc. of 8% gelatin solution, 6 cc. water, 5 cc. borate buffer and 0.5 cc. toluene.

The pH of all the solutions was adjusted at 8.6. Incubation period was 5 hours. The results are given in Table IV.

TABLE IV

Tempera-ture C.	No. of the flask.	Cc. of N/10 NaOH reqd. at the start.	Cc. of N/10 NaOH reqd. after 5 hrs.	Hydrolysis after 5 hrs. in cc. N/10 NaOH.	Hydrolysis due to enzyme in cc. N/10 NaOH.
37°	1.	7.5	10.8	3.3	1.6
	2.	0 4	1.8	1.4	
	3.	2.9	3.2	0.3	
44°	1.	7.5	11.9	4.4	2.6
	2.	0.4	1.9	1.5	
	3.	2.9	3.2	0.3	
50°	1.	7.5	12.3	4.8	2.6
	2.	0.4	2.1	1 7	
	3.	2.9	3.4	0.5	
60°	1.	7.5	13.1	5.6	3.0
	2.	0.4	2.2	1.8	
	3.	2.9	3.7	0.8	
70°	1.	7.5	11.2	3.7	1.0
	2.	0.4	2.2	1.8	
	3.	2.9	3.8	0.9	

The results show that the optimum temperature of the enzyme is near about 60°C.

Effect of KCN, Glutathione, Ascorbic Acid and H_2S on the Activity of the Enzyme.

One g. of the enzyme preparation was made into a cream with water and the final volume was made up to 64 cc.

Flask No. 1 contained 8 cc. of enzyme preparation, 10 cc. of 8% gelatine solution, 5 cc. borate buffer and 0.5 cc. toluene.

Flask No. 2 contained 8 cc. of enzyme preparation, 10 cc. of water, 5 cc. borate buffer and 0.5 cc. toluene.

Flask No. 3 contained 8 cc. water, 10 cc. of 8% gelatine solution, 5 cc. borate buffer and 0.5 cc. toluene.

The pH of the substrate was 8.6 and the flasks were incubated at 37° for 24 hours.

Each set of experiment was divided into two groups, one with 1 cc. of water only and other with 1 cc. solution of 1% KCN, or 0.01% glutathione, of 0.25% ascorbic acid. In the fourth set, the enzyme cream and the gelatine solution were saturated with H_2S for 30 minutes.

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TABLE V

Expt. No.		Flask No.	Cc. of N/10 NaOH reqd. at the start.	Cc. of N/10 NaOH reqd. after incubation for 24 hours.	Hydrolysis after 24 hrs in cc. N/10 NaOH.	Hydrolysis due to the enzyme in cc. N/10 NaOH.
	Without KCN ...	1	7.5	13.5	6	4.5
		2	0.5	1.5	1	
		3	3.1	3.6	0.5	
1.	With KCN ...	1	5.6	12.4	6.8	4.5
		2	0	1.6	1.6	
		3	1.3	2	0.7	
	Without glutathione	1	7.3	13.1	5.8	4.8
		2	0.6	1.4	0.8	
		3	3.3	2.5	0.2	
2.	With glutathione	1	7.4	13.2	5.8	4.8
		2	0.6	1.4	0.8	
		3	2.3	2.5	0.2	
	Without ascorbic acid ...	1	7.3	13.1	5.8	4.8
		2	0.6	1.4	0.8	
		3	3.3	3.5	0.2	
3.	With ascorbic acid ...	1	7.3	13.3	6	4.9
		2	0.6	1.3	0.7	
		3	3.2	3.6	0.4	
	Without H$_2$S ...	1	6.4	10.9	4.5	4.5
		2	1.2	1.2	0.0	
		3	2.8	2.8	0.0	
4.	With H$_2$S ...	1	7.5	12.5	5.0	4.45
		2	2.05	2.3	0.05	
		3	3.6	3.9	0.3	

The results in Table V indicate that KCN, glutathione, ascorbic acid and H$_2$S have no influence on the action of the enzyme.

SUMMARY

· A preparation of a proteolytic enzyme has been obtained from the common white gourd (*Benincasa Criapra*). Its *p*H optimum with Citrate-phosphate buffer and borate buffer with gelatine substrate is on the alkaline side being of the order of 8.6 with borate buffer. Its temperature optimum is of a high order 60°C. The substances like potassium cyanide, glutathione, ascorbic acid and hydrogen sulphide have no influence on the action of the enzyme. These results indicate that this new enzyme is probably different from the other plant proteases so far described.

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REVIEWS

ANNUAL REVIEW OF BIOCHEMISTRY AND ALLIED RESEARCH IN INDIA, Vol. XII *for 1941.* Published by the Society of Biological Chemists, Bangalore, India, July, 1942. Price Rs. 3/- or 6s.

This review is the 12th volume of this annual publication. It covers a wide range of subjects, namely, enzymes, general nutrition, vitamins, proteins, carbohydrate, fat and mineral metabolism, pharmacology, animal nutrition, chemistry of plant products, plant physiology, soil and fertilisers and technical mycology. This review in a nut-shell relates the progress made by Indian workers' research. It serves a very useful purpose by showing in perspective the different types of work that are being carried out and would thereby provide pointers to indicate in which direction research should be pursued in this country. All workers in this field have nothing but praise for the excellent way that this Review is being presented from year to year by the Society of Biological Chemists (India).

CHOLERA: ITS PREVENTION AND CURE. By A. J. H. deMonte, Dip. Bact. (Manchester), I.M.D., Officer-in-Charge, Bowel Diseases Research Department, School of Tropical Medicine, Calcutta. Pp. 60. Price Re. 1/-. Thacker Spink & Co. (1933) Ltd., Calcutta.

This is a useful addition to the growing volume of medical literature of this country and is particularly welcome, as it comes from the pen of one who has got considerable field and laboratory experience of the subject with which he is dealing.

To the scientifically trained medical men who want to be acquainted with the newer knowledge about Cholera, this small booklet will be of immense help. To the English knowing house-holders as well as to those who look after larger groups of people, it will give a very practical and clear, simple and yet scientific guidance when confronted with this severe malady. Apart from the curative treatment in which saline and such controversial agents as potassium permanganate, essential oils, bacteriophage and diseptal B have been cautiously mentioned, the prophylactic methods have been noted in minute details and have occupied a large space. This is not only appropriate but essential especially when dealing with a disease like Cholera whose prevention is so easy but cure is so uncertain. At least this feature alone distinguishes this small booklet from the ordinary run of text-books on Cholera. We particularly recommend it to the social service workers, and to those responsible for the maintenance of health of large groups of people in regions where Cholera occurs endemically or in epidemics.

ASCORBIC ACID VALUE OF INDIAN GOOSEBERRY
(*PHYLLANTHUS EMBLICA*)

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The importance of vitamin C in nutrition has been increasingly realised within recent years. With the advent of the present war the supply of vitamin C to the fighting units from easily available sources has also engaged the attention of scientific workers. About seven years ago Damodaran and Srinivasan (1) were the first to report the richness of the fruits of *Amla* or Indian gooseberry (*Phyllanthus emblica*) in ascorbic acid. This observation was confirmed three years later by Giri and Doctor (2). In a further communication Giri (3) discussed the potency of ascorbic acid as affected by storage as dry powder or as pickles. In the present investigation an attempt has been made to study the ascorbic acid content of fresh samples of *amla* berries obtained from various parts of the province of Bihar to arrive at an average ascorbic acid content figure and also to record the variations in potency caused by different methods of storage for fairly long periods.

EXPERIMENTAL

As soon as a consignment of fruits was received, a few fresh berries were picked out, washed clean of extraneous matter, the moisture was wiped off with the help of dry linen and the berries were finally dried under the fan. The fruits were sliced and random samples of slices were collected for three separate estimations. Ascorbic acid was estimated with 2:6 dichlorophenolindophenol in accordance with the technique described in a previous communication from this laboratory (4). The ascorbic acid content of each sample was recorded from the average of three estimations.

For making powder from the fruits fresh samples of berries were mashed in a curry stone, the seeds removed and the pulp ground to paste. The softened pulp was then spread over rush mat and dried in the shade. After drying, the dried lump was scraped out and finely powdered in a stone mortar. After thorough grinding the contents of the mortar were strained through cloth and the powder reduced to the consistency of milled whole wheat flour. The residue on the strainer (particles about the size of massoor or lentil) was stored in cigarette tins. Finally the strained powder was stocked in clean glass stoppered phials, pasted all round and over the top with black paper. Immediately before estimations of ascorbic acid were made, the bottle was thoroughly shaken and the contents stirred with a horn spatula so as to ensure thorough mixing and representative samples being taken out for analysis. The estimations were made in the same way as in the case of pulp of the fresh fruits. As the moisture content of the different samples and of the same sample at different stages of experiment was found to vary, the ascorbic acid content on dry weight of the respective samples has been calculated.

TABLE I

Ascorbic acid content of fresh samples of 'amla'.

Serial No.	Source (District)	Dates of analysis.	Ascorbic acid mg./100 g. of pulp.	Moisture %.	Ascorbic acid mg./100 g. of sample (dry weight)
1.	Patna	13.2.41	281.9	81.9	1553.2
2.	Manbhum	20.2.41	369.6	83.3	2215.8
3.	Palamau	23.2.41	307.3	78.7	1439.3
4.	Hazaribagh	1.3.41	320.4	80.1	1610.8
5.	Singhbhum	15.3.41	346.7	78.5	1610.3
6.	Shahabad	17.3.41	419.1	80.6	2162.5
7.	Ranchi	7.4.41	275.9	78.3	1223.6
8.	Ranchi	5.5.41	453.2	77.6	2024.8

RESULT AND DISCUSSION

From Table I it appears that per g. of fresh pulp the ascorbic acid content varied from 2.8 to 4.5 mg. with an average of 3.5 mg. Thus these figures are more in keeping with the observations recorded by Damodaran and Srinivasan (1) than with those of Giri (3) who has recorded a comparatively high average figure of 7.2 mg. of ascorbic acid per 1 g. of fresh pulp. One of the explanations for comparatively low figures observed in the present series might be due to the fact that the ascorbic acid potency of the berries grown in this part of the country is lower than that of berries grown in the other part (Nilgiris) where Giri received his supplies from.

Moreover, the fruits were subjected to analysis at least 4 days after they were plucked from the tree and in any case not later than 8 days. This time was taken for the transport of the fruits from their respective sources to the laboratory by railway parcel. It is quite within the bounds of possibility that if the analyses were made within 2 days of plucking higher figures would have been obtained. Probably as a result of the combined effects of both the factors such low figures were obtained. The difference in moisture percentage is not likely to be responsible for a discrepancy of this order.

The comparative potency of ascorbic acid as affected by storage of dry powder in refrigerator and at room temperature is shown in Table II.

TABLE II

Ascorbic acid content of 'amla' powder (calculated on dry weight of sample) affected by storage at different temperatures.

Dates of estimation.	Period of storage in days.	In refrigerator			At room temperature 21°—36°		
		Moisture %.	Ascorbic acid mg/100 g.		Moisture %.	Ascorbic acid mg/100 g.	
			Actual.	%.		Actual.	%
4.4.41	0	10.5	1247.7	100.0	10.5	1247.0	100.0
6.5.41	32	11.1	1224.9	98.2	9.6	967.0	77.5
6.6.41	63	10.1	1047.8	84.0	9.7	838.6	67.2
6.7.41	93	10.1	1045.1	83.8	9.4	798.4	64.0
29.10.41	208	10.3	1025.2	82.2	9.1	616.6	49.4
14.2.42	316	10.2	1016.8	81.5	9.1	579.6	46.5
14.4.42	375	10.2	1008.6	80.8	9.1	419.6	33.6

Storage in refrigerator.—The sample preserved in the refrigerator thus maintained a higher potency than that kept at room temperature and this was expected. Giri (3) noticed no deterioration in ascorbic acid content in *amla* powder even after a storage of three months in the refrigerator at 0°C, but the experience in the present investigation has been otherwise. A deterioration of about 16% in potency was noticed in the sample stored inside the refrigerator the temperature of which was found to vary from 5°C to 7°C. Probably the slightly higher temperature of the refrigerator used in the present investigation was responsible for the loss. Another interesting feature noticed was that in the course of next ten months the loss in the ascorbic acid potency was barely 4% as compared with the corresponding loss (16%) in the first two months.

In order to find whether coarse powder keeps better than fine powder the residue from straining (of the size of lentils) preserved in the cigarette

tin mentioned previously was stored in the refrigerator and analysed. It was found that after a period of 208 days the ascorbic acid content was reduced from 1247.7 to 1155.5 mg./100 g. (calculated on dry weight) ; in other words a loss of 9.3% only as compared with the corresponding loss of slightly less than 18% observed in the powder of finer consistency. It is to be regretted that through inadvertance this tin was kept out of the refrigerator and observations could not be continued till the end of the period of enquiry.

A small number of *amla* tablets (prepared from the dried pulp) were very kindly lent by Dr. W. R. Aykroyd, Director, Nutrition Research Laboratories, Coonoor. On 11.8.41 the ascorbic acid content was determined as 962.2* mg./100 g. on the average of 3 estimations as usual and the tablets stored in the refrigerator. Representative samples were analysed on 29.10.41 (after 79 days), 14.2.42 (after 188 days) and on 14.4.42 (after 247 days) and the corresponding figures were 913.3,* 882.0* and 875.4* mg./100 g. of ascorbic acid, thus indicating a loss of about 9 per cent. during the whole period. From the above findings it would not be wrong to presume that under similar conditions of storage coarser grains lost their potency of ascorbic acid more slowly as compared to finer grains prepared from the dried pulp of *amla*. Per unit of weight bigger particles of coarse powder have lesser surface area exposed to the air as compared with powder of finer division. Alston (5) states that during storage in air-tight tins even the minimal residual air acts adversely on the ascorbic acid potency.

Storage at room temperature.—In the case of (fine) powder stored at room temperature the ascorbic acid potency was reduced by one-third in about two months' time at the beginning and an equal amount of loss in potency was observed in the next ten month period. In the case of the sample stored in the refrigerator the loss in potency observed during the first two months of storage has been comparatively more rapid. Freshly prepared powder has a creamy whitish colour and with deterioration in potency the colour was found to change to brown, the depth of which varied according to the degree of loss in potency.

Action of preservatives—In order to investigate the stability of ascorbic content of *amla* with various preservatives, a certain number of fresh fruits were (*a*) thickly coated with paraffin, (*b*) steeped in saturated solution of common salt (prepared at room temperature, a slight excess being added) and stored in a glass jar, (*c*) similarly steeped in thick syrup solution of cane sugar (containing about 350 parts of sugar per 100 parts of water by weight) and (*d*) in honey obtained locally. The fruits coated with paraffin started discoloration which was visible through the coating in about four weeks' time and the inside was completely disintegrated and liquefied in about 9 weeks' time. Consequently estimation of ascorbic acid content was not attempted.

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*Calculated on dry weight basis.

TABLE III

Ascorbic acid potency of fresh berries stored with different preservatives
(calculated in mg./ 100. g. dry weight)

Period of storage in days.	Common salt.		Sugar syrup.		Honey.	
	Moisture %.	Ascorbic acid (mg.)	Moisture %.	Ascorbic acid (mg.)	Moisture %.	Ascorbic acid (mg.)
0	81.9	1553.2 100%	80.1	1610.8 100%	80.1	1610.8 100%
31	63.1	706.7 45.5%	41.3	668.7 41.5%	30.5	1093.3 67.9%
62	65.6	702.6 45.2%	33.0	355.9 22.1%	27.7	505.9 31.4%
93	64.7	312.6 20.1%	32.3	346.8 21.5%	28.5	436.9 27.1%
120	67.6	8.5%	33.7	190.2 11.8%	28.7	394 5 24.5%
208	68.2	Trace	36.1	181.7 11.3%	29.7	391.7 24.3%

Referring to the ascorbic acid potency with different liquid preservatives (see Table III) it is found that saturated common salt solution, sugar syrup or honey does not exert any appreciable preservative action though one had expected better results with the first named preservative in view of the observations made by Høygaard and Rasmussen (6) that sodium chloride even in 1% solution inhibits the oxidation of ascorbic acid during the process of cooking. Out of the three preservatives used honey seemed to be the best as even after 7 months the ascorbic acid content was found to be higher than those of the berries stored in the other two liquids. The berries preserved in common salt, however, developed slightly pleasant taste, but those in honey or sugar syrup retained the astringent taste present in the fresh fruits. These findings justify the tentative conclusion that in dry powder of the berry ascorbic acid is more stable than in fresh fruits preserved in liquids.

SUMMARY

The potency of ascorbic acid in the pulp of fresh *amla (Phyllanthus emblica)* fruits obtained from eight different localities was found to vary between 2.8 and 4.5 mg. per g. by using Tillman's dye titration method. Powder made from dried pulp of the berries was found to lose about 20% of its ascorbic acid content when kept in a refrigerator for 375 days. The

corresponding loss in the same sample of powder kept at room temperature was found to be as high as 67% of the total. In the fresh berries preserved in common salt solution, sugar syrup or honey more than three-fourths of the ascorbic acid disappeared in about 3 months', 2 months' and 4 months' time respectively.

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NICOTINIC ACID CONTENT OF BLOOD IN DISEASES.
PART I. ANÆMIA.

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It has been shown (2) that the numerical strength of erythrocytes and leucocytes can be a factor influencing the nicotinic acid content of blood. The nicotinic acid content of blood will be low when the cell volume of the blood falls. This explains the low nicotinic acid content of the blood in anæmia as observed previously (2). In dogs though the cell volume is lower than that of man the nicotinic acid content of the blood is higher (3). This discrepancy in dogs may be explained by the high leucocytic count which is roughly thrice that of man. In some cases showing leucocytosis, a slight increase in nicotinic acid content of blood was noted (2). It would thus appear that the relative number of erythrocytes and leucocytes determines the nicotinic acid content of blood. Nicotinic acid is a part of coenzymes I and II, the concentration of which in cells in pellagra was shown to be only moderately low by Kohn and Bernhein (4). Further Vilter, Koch and Spies (8) have observed that the degree of deficiency of coenzymes I and II in whole blood and in packed cells was not parallel to the severity of any one of the signs of pellagra. The total coenzyme value per cc. of blood cells is higher in anæmia than in a normal subject. Similar observations with respect of nicotinic acid in anæmia have been made by Melnick, Robinson and Field (6). It may be assumed that part of the decreased nicotinic acid content due to low cell volume in anæmia is made up by the increased concentration of nicotinic acid in cells and that the total amount of nicotinic acid in whole blood is but little affected. This assumption is in concordance with the conclusions reached in a previous communication (2) that the fluctuation of blood nicotinic acid values in patients suffering from various diseases including pellagra was insignificant. In the pellagrin the nicotinic acid content of blood fell within the normal limits and no obvious rise of nicotinic acid content

occurred as a result of nicotinic acid therapy. In normal persons about 10 per cent of nicotinic acid was shown to be present in plasma and the rest 90 per cent in the blood cells. In anæmia where the blood nicotinic acid falls slightly, about 90 per cent of blood nicotinic acid was shown to be present in cells by Melnick et al (6). Thus both in normal and diseased states the concentration of nicotinic acid in blood shows practically no significant difference. Another reason for fixidity of the concentration of nicotinic acid in blood may be adduced from the observation of Kohn, Klein and Dann (5) that the coenzyme of liver and striated muscles was greatly reduced in black tongue but that in tissues such as brain and kidney was unaffected. These results, which were confirmed by Axelrod and his associates (1) suggest that the liver was sacrificing its store of coenzymes to maintain the level of coenzymes in other tissues. This has a parallel in rickets and osteomalacia where the calcium content of the blood is not markedly affected until late in the disease.

These facts are of considerable interest and it may be assumed that the nicotinic acid content of blood, like that of coenzymes I and II, may parallel the morphological characteristics of the blood rather than the severity of the disease. To test this hypothesis investigations were undertaken to discover variations of nicotinic acid content in the various constituents of blood in disease and attempts have been made to correlate clinical symptoms with other laboratory findings. Since nicotinic acid in blood is mainly present in blood cells, the volume of the cells is a factor which may influence the nicotinic acid content of blood. Two variables—cell volume and hæmoglobin—were studied with view to establish a correlation between them and the nicotinic acid content.

RESULTS AND DISCUSSION

The nicotinic acid content of blood in 30 cases of anæmia is shown in Table I, the average being 326 μg. per cent ranging from 199 to 480 μg. per cent. This average, though a little lower than that of normal persons, falls (except for one case) within the lower range of normal distribution. The nicotinic acid content of plasma from these cases was also examined. The average nicotinic acid content of plasma in these cases was 131 μg. per cent whereas in normal subjects it was less than 100 μg. per cent. The probable explanation of this discrepancy may be that the ratio between the nicotinic acid concentration in cells and in plasma tends to remain constant. Plasma values may be high in cases of anæmia of the hæmolytic type due to liberation of nicotinic acid from the disintegrating erythrocytes, but this suggestion lacks experimental verification. On the other hand, the only case of hæmolytic anæmia examined (Table I, No. 2), showed plasma values much below the average.

TABLE I
Nicotinic Acid content of blood in anæmia.

No.	Case No.	Packed Cell Volume %.	M. C. V. Cu.μ	Hæmoglobin %.	M. C. H. γγ.	M. C. H. C. %.	Nicotinic acid. Blood μg %.	Plasma μg %.	Packed cells μg %.	% blood nicotinic acid in packed cells.
I.	31	7.0	96	12	27.4	29	252	101	1888	59.6
2.	3	9.5	95	25	41.6	44	248	100	1658	63.5
3.	34	8.0	109	14	32.0	29	320	158	1778	55.5
4.	47	11.0	71	27	31.7	41	199	90	1072	59.7
5.	36	12.0	164	—	—	40	280	200	886	38.0
6.	23	12.0	93	35	45.2	49	304	135	1543	60.9
7.	9	12.0	162	19	42.7	26	307	141	1529	59.6
8.	53	12.0	266	25	91.1	34	228 +	150	1640*	60.0
9.	54	12.0	78	18	19.3	25	388 +	100	2400*	74.2
IO.	14	12.2	163	18	40.0	25	428	223	1903	54.2
II.	44	12.2	93	30	37.8	40	428	220	1925	54.8
I2.	18	12.3	70	29	27.4	39	—	280	—	—
I3.	45	13.0	—	—	—	—	312	94	1694	70.6
I4.	46	14.3	75	35	30.5	40.8	248	115	930	53.6
I5.	7	15.0	136	—	—	—	330	207	1027	46.7·
I6.	28	15.9	80	30	25.2	31.4	392	129	1780	72.6
I7.	10	16.0	82	20	17.1	21.0	312	186	974	50.0
I8.	29	16.0	126	32	43.1	33.3	334	80	1667	79.8
I9.	42	16.0	81	25	47.3	25.9	372	107	1663	71.5
20.	17	16.4	166	22	37.8	22.6	320	100	1442	73.9
2I.	48	18.0	66	60	36.3	55.5	215	65	898	75.1
22.	55	18.0	80	23	16.8	21.0	233 +	140	780*	62.2
23.	2	18.5	87	40	31.3	36.0	376	95	1664	81.9
24.	35	19.2	91	40	31.3	34.7	400	120	1578	75.6
25.	8	26.9	112	52	36.1	32.2	355	83	1019	77.2
26.	4	28.4	131	55	42.4	32.2	364	90	1055	82.3
27.	30	28.5	79	55	25.4	32.1	392	172	944	69.4
28.	24	35.0	61	90	26.0	43.0	480	123	1143	83.3
29.	I	—	6	—	—	—	400	30	—	—
30.	7	—	—	—	—	—	448	118	—	—
Average.		15.9±7.0					326	131	1426±165	

NOTE:— + Calculated.
      * Experimental.
      M. C. V. =Mean cell volume=
                  Volume of packed cells in cc. per 100 cc. of blood
                  ——
                  Red cell count (in millions per c.m.)
      M. C. H. =Mean corpuscular hæmoglobin=
                  G. of hæmoglobin per 1000 cc. of blood
                  ——————————————————————————————————
                  Red cell count (in millions per c.m.)
      M. C. H. C. =Mean corpuscular hæmoglobin concentration=
                  G. of hæmoglobin per 100 cc. of blood
                  —————————————————————————————————
                  Volume of packed cells, cc. per 100 cc. of blood
   Cell counts, hæmoglobin determination and most of the hæmatocrit values
      were done by the Mayo Hospital staff.

2

Table II A suggests that a low cell volume was associated with a decrease in nicotinic acid content of blood together with high concentration of cell nicotinic acid and *vice versa*.   It may be inferred that a high concentration of nicotinic acid in cells compensates for the decrease of nicotinic acid resulting from their low numerical strength.   The values for blood in anæmia are, therefore, only slightly affected.   This means that the concentration of nicotinic acid per cell increases with the fall of cell volume.   Hæmoglobin, the quantity of which generally decreases in anæmia, seems to have only a little effect on the concentration of nicotinic acid in blood cells.   Further in the same table it has been shown that in anæmia the average nicotinic acid values of blood are dependent only slightly on the mean corpuscular volume of the cell.   Table II also shows that mean corpuscular hæmoglobin and mean corpuscular hæmoglobin concentration had little or no effect on the concentration of nicotinic acid in blood cells.   Charts 1 and 2 show the relation of nicotinic acid to packed cell volume and hæmoglobin respectively.

TABLE II
Correlation between nicotinic acid values and the blood variables.
A.   Packed Cell Volume.

Packed cell volume. %	Total No. of cases.	Mean packed cell volume. %	Average nicotinic acid.		
			Blood μg %.	Plasma μg %.	Cells μg %.
Normal		42	417	100	850*
Greater than 30	1	35	480	123	1143
20 to 30	3	27.9	370	115	1006
15 to 20	9	17.1	385	113	1383
10 to 15	11	12.5	323	163	1506
Less than 10	3	8.2	273	120	1775

B.   Hæmoglobin.

Hæmoglobin. %	Total No. of cases.	Average Hæmoglobin. %	Average nicotinic acid.		
			Blood μg %.	Plasma μg %.	Cells μg %.
Normal		90	417	100	850*
Greater than 50	5	62.0	361	106	1010
25 to 50	10	31.9	330	119	1548
Less than 25	9	19.0	323	144	1593

C.   Mean Corpuscular Volume (M.C.V.)

M. C. V. cu. μ	Total No. of cases.	Average M. C. V. cu. μ	Average nicotinic acid.		
			Blood μg %.	Plasma μg %.	Cells μg %.
Greater than 94	12	144	322	136	1458
94 to 80	8	87.7	352	141	1463
Less than 80	5	73.6	288	108	1249

D.   Mean Corpuscular Hæmoglobin (M.C.H.)

M. C. H. γγ	Total No. of cases.	Average M. C. H. γγ	Average nicotinic acid.		
			Blood μg %.	Plasma μg %.	Cells μg %.
Greater than 33	12	45.1	333	124	1495
25 to 33	9	29.3	324	127	1321
Less than 25	3	17.7	311	142	1385

E.   Mean Corpuscular Hæmoglobin Concentration (M.C.H.C.).

M. C. H. C. %	Total No. of cases.	Average M. C. H. C. %	Average nicotinic acid.		
			Blood μg %.	Plasma μg %.	Cells μg %.
Greater than 38	7	44.6	306	121	1310
30 to 38	8	33.2	368	115	1418
Less than 30	9	23.9	325	139	1595

*Calculated.
†100% Hæmoglobin is taken as 16.6 g. per 100 cc.

CHART 1.

Correlation of hæmatocrit values to concentration of nicotinic acid in blood constituents.
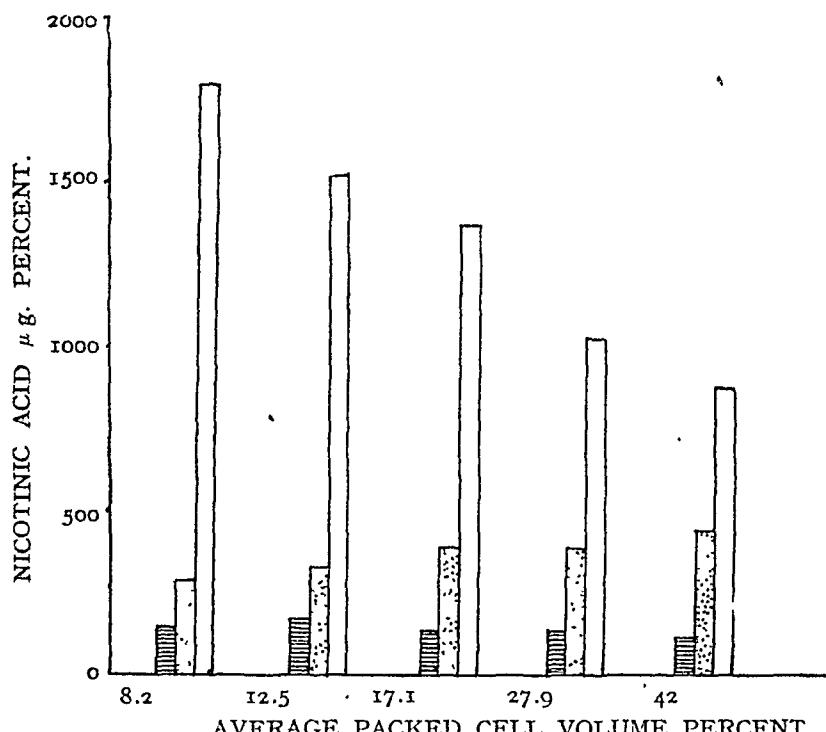
CHART 2.

Correlation of hæmoglobin to concentration of nicotinic acid in blood constituents.
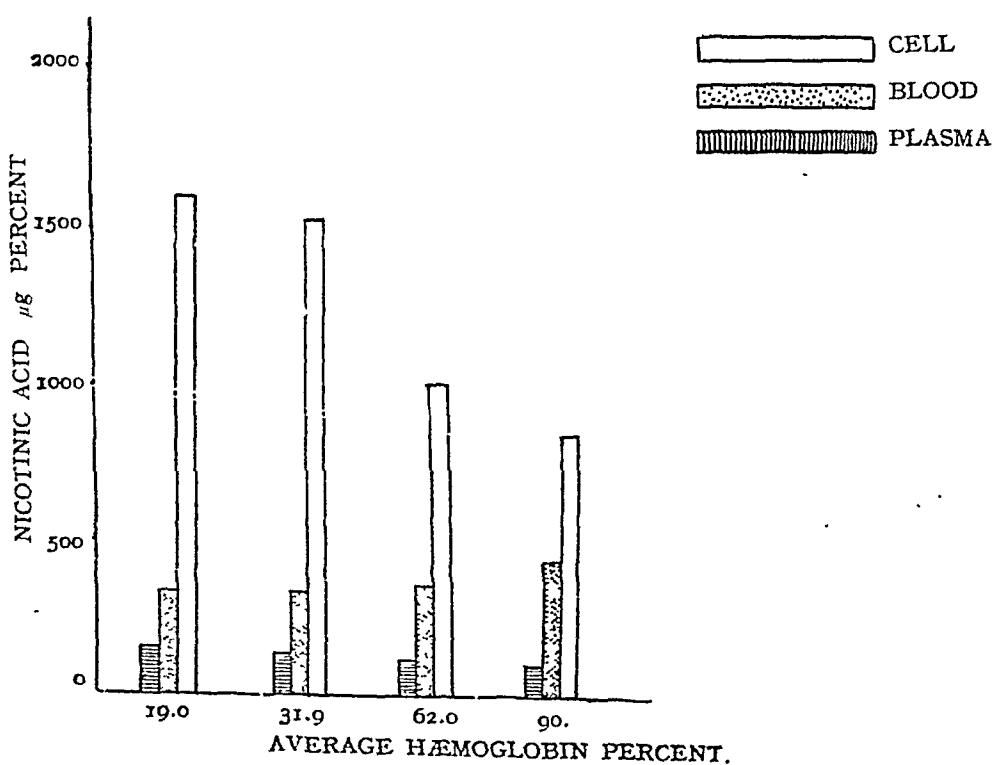
TABLE III

Packed cell volume %.	Nicotinic acid in cell.			Total No. of cases.	Percentage.
	Less than 1000.	1000 to less than 1500.	1500 to less than 2000.		
Less than 10	—	—	3	3	12
10 to less than 20	5	4	10	19	76
20 to less than 30	1	2	—	3	12
Total No. of cases	6	6	13	25	—
Percentage	24	24	52	—	—

Table III suggests that with low packed cell volume the nicotinic acid concentration in cell is high and *vice versa*. The coefficient of correlation between cell volume and nicotinic acid of cells was calculated for all the 25 anæmia cases treated as one sample and the value obtained was 0.56 which is about 2.8 times of its standard error $1 \sqrt{25-1}$ It is, therefore, statistically established that the nicotinic acid content of blood is not independent of cell volume and the two are inversely related. But the coefficient is not sufficiently high to establish any clinical significance. The value X^2 for this table was found to be 7.5. This value when referred to Fishers' table for 4 degree of freedom gives a probability figure of 0.12 showing that the difference between the proportions of cases in each cell of this table might have arisen by chance and we can draw from only tentative conclusions from these results. It is quite clear from the scatter diagram, (graph 1), that the degree of correlation is not sufficiently high to make the test of any use in clinical practice.

TABLE IV

Hæmoglobin %.	Nicotinic acid in cell.			Total No. of cases.	Percentage.
	Less than 1000.	1000 to less than 1500.	1500 to less than 2000.		
More than 50	2	3	—	5	21.7
25 to 50	1	1	8	10	43.5
Less than 25	2	1	5	8	34.8
Total No. of cases	5	5	13	23	—
Percentage	21.7	21.7	· 56.6	—	—

GRAPH. 1.
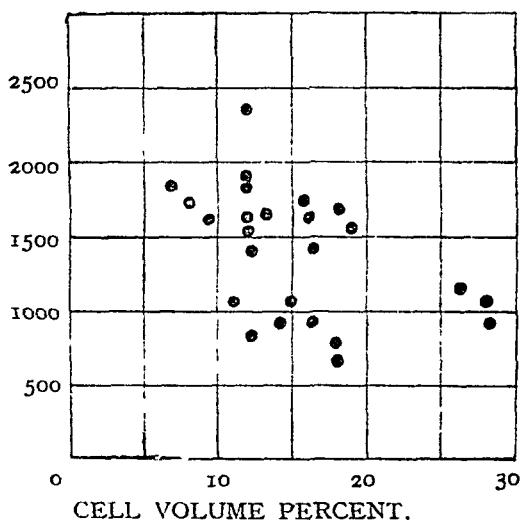
GRAPH. 2.
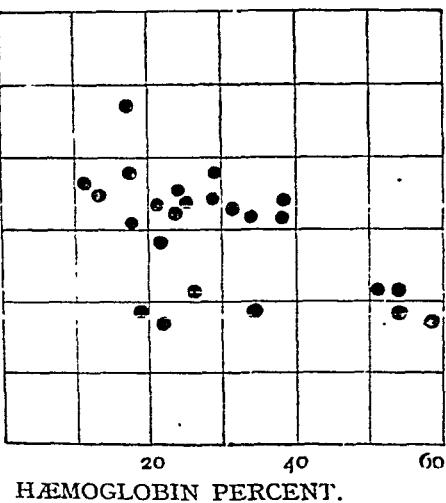
Table IV suggests that with low hæmoglobin the nicotinic acid concentration in cells is high and *vice versa*. The value X^2 for this table was found to be 12.86. This value when referred to Fishers table for 4 degree of freedom gives a probability figure 0.016, establishing a significant degree of association between the two variables. The value of co-efficient of correlation for these 23 cases is -0.38 which is about 1.86 times of its standard error of $\frac{1}{\sqrt{23-3}}$ showing no significant degree of association. Graph 2 confirms the latter inference.

The following classification of anæmia into 4 groups, according to size and hæmoglobin content of cells, has been proposed by Wintrobe (9).

	M. C. V. cu. μ.	M. C H. C. %.
1. Macrocytic	Greater than 94	Greater than 30
2. Normocytic	80—94	,, ,, 30
3. Simple Microcytic	Less than 80	,, ,, 30
4. Hypochromic Microcytic	Less than 80	Less ,, 30

This classification is based on the cell volume and hæmoglobin which seem to possess statistically no correlation with the concentration of nicotinic acid in blood or in cells. Nor does the perusal of the above tables indicates any such association. On the other hand it shows that the content does not parallel the type or the severity of the disease. Thus blood is not necessarily the best indicator of pathological processes in the tissues for nicotinic acid deficiency. In the course of this work it has already been shown (2) that the estimation of nicotinic acid content of blood is probably of little value in the diagonosis of nicotinic acid deficiency. As has been observed, that decreased nicotinic acid content due to low hæmatocrit value in anæmia is made up by the increased concentration of nicotinic acid in cells (mean 1426 μg. per cent.) and the sum total of nicotinic acid in whole blood is but little affected. The possible reason for the increased concentration of nicotinic acid in cells in anæmia may be the nature's adjustment to supply coenzymes to the tissues at increased rate due to the increased utilization of oxygen by them. Tissues in anæmia become gradually adapted to lower oxygen tension as the reduction of hæmoglobin progresses (Richard and Strauss, 7). It can be conceived that a diminution of the supply of erythrocyte to the tissues will, on account of the limitation of oxygen and the coenzymes, otherwise, result in the most profound disturbances of the chemical reactions which underlie tissue functions.

SUMMARY

(1) The nicotinic acid content of the various constituents of the blood was determined in 30 cases of anæmia classified according to size and hæmoglobin content of cells.

(2) In anæmia the decreased cell volume was accompanied by a decrease in the nicotinic acid content of blood together with an increase in the nicotinic acid concentration of the cells whereas the total nicotinic acid of the whole blood was slightly lowered..

(3) It has been experienced that in anæmia the concentration of nicotinic acid in blood does not parallel the type or the severity of the disease.

*Calculated.
†109% Hæmoglobin is taken as 16.6 g. per 100 cc.
    Correlation of hæmatocrit to concentration of nicotinic acid in blood.
    Correlation of hæmoglobin to concentration of nicotinic acid in blood.

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THE EFFECT OF NARCOTICS ON THE EXCRETION OF FREE AND COMBINED ASCORBIC ACID.

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Earlier (1, 2) it was observed that the administration of diphtheria and tetanus toxins caused increased elimination of combined ascorbic acid in the urine of guinea pigs and the diminution of free ascorbic acid excretion. Later (3) it was observed that when quite different toxic substances like histamine, camphor, chloralhydrate. phenol, phosphorus, copper, fluoride and cyanide were administered, the amounts of free and combined ascorbic acid excretion varied depending on the nature of the toxic material employed.

3

Recently, Longenecker, Musulin, Tully and King (4) have observed increased elimination of free ascorbic acid in the urine of rats when fed with a great variety of organic substances including nerve depressants (5). These investigators were, however, unable to observe any elimination of combined ascorbic acid in the urine of both normal and narcotised rats. The methods employed by these investigators were, however, not those followed by us. The present investigation has been carried out in order to observe the effect of certain narcotics on the urinary excretion of both free and combined ascorbic acid in two different species of animals namely, the rat and the guinea pig, by the method of estimation developed in these laboratories. Tissue ascorbic acid has also been estimated after administration of the narcotics. These investigations are necessary in order to throw more light on the appearance of combined ascorbic acid in the urine and also to know by what process the increased excretion of free ascorbic acid is brought about in the narcotised rats.

EXPERIMENTAL

Both rats and guinea pigs were employed in the present investigation. Each of the animals was kept separately in a metabolism cage and the urinary excretion of ascorbic acid (both free and combined) was determined by the method already described (1-2).

Rats between the weights 150 and 200 g. were kept on a normal diet of whole wheat, brown bread and milk. Guinea pigs were fed on green grass and germinated gram and their weights varied from 250 to 300 g. Narcotics like chloretone, paraldehyde, phenobarbital, urethane, amidopyrine and bromobenzene were fed to the experimental animals. The results obtained with the two different species are given in Tables I and II.

It will be seen from Table I that the administration of chloretone paraldehyde, phenobarbital, amidopyrine and bromobenze caused an increased elimination of free ascorbic acid in the urine of rats. The excretion of combined ascorbic acid was markedly reduced especially in cases where chloretone, amidopyrine, and bromobenzene were fed.

From Table II it will be seen that the administration of paraldehyde, amidopyrine and bromozenbene and, to a certain extent, of chloretone influenced similarly the elimination of free ascorbic acid in the urine of

guinea pigs. The administration of urethane caused an increased excretion of both free and combined ascorbic acid. Phenobarbital caused decreased elimination of both free and combined ascorbic acid in the urine. The animals experienced severe shocks when phenobarbital was administered.

A study of the amounts of ascorbic acid present in the brain, liver and kidney tissues of the narcotised rats and in the adrenal, liver and kidney tissues of guinea pigs revealed very striking differences (Table III). A general depletion of tissue ascorbic acid was observed in guinea pigs even when they were able to excrete increased amounts of free ascorbic acid. Rats, on the other hand, showed increase both in the concentration of tissue ascorbic acid and of urinary free ascorbic acid.

It is, therefore, possible to conclude that the mechanism of increased excretion of free ascorbic acid in the rat followed a different path and in the intact animal increased synthesis of ascorbic acid takes place but in cases of guinea pigs, the increased elimination of free ascorbic acid in the urine was due to the disposal of body reservoir of ascorbic acid.

The existence of combined ascorbic acid in the urine has been questioned by Longenecker, Frisk and King (5) who were unable to detect the presence of combined ascorbic acid in the urine of rats. These investigators have not, however, followed the method of Sen-Gupta and Guha (6) or of Scarborough and Stewart (7). Moreover, these investigators employed the urine of chloretone fed rats for the estimation of combined ascorbic acid. The urine of such rats contains, in fact, no combined ascorbic acid, as would be evident from the results given in Table I.

The complete disappearance of combined ascorbic acid from the urine of the chloretone-fed rats may perhaps be related to the complete saturation of the body with ascorbic acid whose synthesis is apparently increased enormously in this condition. Administration of 50 mg. ascorbic acid to normal rats caused similar disappearance of combined ascorbic acid (Table IV). Similar disappearance of combined ascorbic acid from the urine of normal and tuberculous patients on being dosed with vitamin C has been reported by Banerjee and Guha (8).

The results of a number of experiments showing the existence of both free and combined ascorbic acid in the urine of rats when fed with a large variety of substances are tabulated in Table V.

TABLE I

Mg. of ascorbic acid excreted per animal during a period of 4 days before and after administration of the following substances.

No.	Nature and quantity of the material.	No. of cases.	Free ascorbic acid.		Combined ascorbic acid.	
			Before.	After.	Before.	After.
I.	Chloretone (20 mg.) dissolved in 0.1 cc. coconut oil	1	1.05	4.08	0.43	0
		2	1.37	4.95	0.36	0
		3	0.93	4.08	0.44	0
		4	0.15	4.72	0.40	0
		5	0.40	3.25	0.42	0
II.	(a) Paraldehyde (20 mg.) in 0.1 cc. water as emulsion.	1	0.36	0.70	0.40	0.23
		2	1.37	2.70	0.30	0
		3	1.03	1.47	0.57	0.12
		4	0.61	1.32	0.42	0
	(b) Paraldehyde (0.1 cc.)	1	0.90	1.86	0.53	0.44
		2	0.61	1.14	0.48	0.23
		3	0.61	0.82	0.46	0.26
		4	0.73	1.33	0.32	0.23
III.	Phenobarbital (20 mg.) dissolved in bicarbonate solution (0.1 cc.)	1	0.31	0.80	0.65	0.63
		2	0.26	1.26	0.43	0.39
		3	0.19	1.24	0.57	0.13
		4	0.28	0.93	0.63	0.04
IV.	Amidopyrine (20 mg.) in 0.1 cc. 5% alcohol.	1	0.44	1.59	0.48	0
		2	0.56	1.83	0.63	0
		3	0.41	2.25	0.41	0
		4	0.79	1.24	0.26	0
		5	1.15	1.53	0.28	0
V.	Urethane (100 mg.) in 0.1 cc. water.	1	0.61	0.40	0.33	0.58
		2	0.75	0.68	0.30	0.37
		3	0.70	0.61	0.35	0.58
		4	0.67	0.54	0.37	0.74
		5	0.65	0.54	0.14	0.28
VI.	Bromobenzene (0.1 cc.)	1	0.54	0.71	0.26	0
		2	0.61	0.70	0.09	0
		3	0.47	0.73	0.28	0
		4	0.90	1.01	0.30	0.13
		5	0.44	0.90	0.41	0.14

TABLE II

Mg. of ascorbic acid excreted per guinea pig for a period of 3 days before and after administration of the following substances.

No.	Nature and quantity of the material.	No. of cases.	Free ascorbic acid.		Combined ascorbic acid.	
			Before.	After.	Before.	After.
I.	Choloretone (20 mg.) in cocanut oil (0.1 cc.)	1	0.49	0.78	0.10	0
		2	0.90	0.90	0.23	0
		3	0.51	0.61	0.40	0
		4	0.38	0.44	0.18	0.05
		5	0.52	0.54	0.09	0
		6	0.56	0.67	0.03	0
II.	(a) Paraldehyde (0.1 cc.)	1	0.67	0.87	0.32	0
		2	0.59	0.79	0.11	0
		3	0.75	1.12	0.65	0
		4	0.41	0.42	0.23	0.04
	(b) Paraldehyde (0.2 cc.)	1	0.72	1.20	0	0
		2	0.47	0.53	0.17	0.11
		3	0.66	0.68	0.23	0.07
III.	Phenobarbital (20 mg.) dissolved in warm $NaHCO_3$ solution (0.1 cc.)	1	0.55	0.31	0.18	0.07
		2	0.49	0.33	0.24	0.05
		3	0.30	0.21	0.22	0.19
		4	0.25	0.25	0.37	0.00
		5	0.24	0.21	0.21	0.10
IV.	Amidopyrine (50 mg.) in 50% alcohol (0.25 cc.)	1	0.32	1.15	0.13	0.41
		2	0.17	2.10	0.15	0.57
		3	0.27	1.37	0.13	0.37
		4	0.23	1.56	0.26	0.70
		5	0.40	1.40	0.22	0.37
V.	Urethane (100 mg.) in 0.1 cc. water.	1	0.61	0.80	0.35	0.50
		2	0.42	0.68	0.23	0.37
		3	0.42	0.68	0.12	0.19
		4	0.54	0.79	0.11	0.19
		5	0.49	1.02	0.19	0.20
VI.	Bromobenzene (0.1 cc.)	1	0.47	0.59	0	0
		2	0.83	1.11	0.18	0
		3	0.31	0.96	0.09	0
		4	0.47	0.73	0.10	0.07
		5	0.60	1.01	0.13	0

TABLE III
Tissue ascorbic acid.

(a) *In the guinea pig.*

No.	Nature and quantity of substance administered .	Mg. of ascorbic acid per g. of the fresh tissue.		
		Adrenal.	Liver.	Kidney.
1.	Chloretone (20 mg.)	0.176	0.073	0.047
2.	Phenobartital (20 mg.)	0.151	0.081	0.047
3.	Paraldehyde (20 mg.)	0.174	0.069	0.043
4.	Amidopyrine (20 mg.)	0.318	0.088	0.047
5.	Urethane (100 mg.)	0.219	0.104	0.169
6.	Bromobenzene (0.1 cc.)	0.127	0.067	0.046
7.	Normal controls	0.326	0.197	0.088

(b) *In the rat.*

No.		Brain.	Liver.	Kidney.
1.	Chloretone (20 mg.)	0.338	0.279	0.252
2.	Paraldehyde (0.1 cc.)	0.310	0.251	0.173
3.	Phenobarbital (20 mg.)	0.259	0.224	0.181
4.	Amidopyrine (20 mg.)	0.285	0.283	0.243
5.	Normal control	0.316	0.185	0.164

TABLE IV
Disappearance of combined ascorbic acid from the urine of ascorbic acid- and chloretone-fed rats.

No. of Experiment.	Substance administered.	Mg. of ascorbic acid excreted per animal for a period of 4 days.			
		Free ascorbic acid.		Combined ascorbic acid.	
		Before.	After.	Before.	After.
I.	50 mg. ascorbic acid injected interperitoneally daily	1.03	13.65	0.45	0
II.	,,	1.28	14.30	0.47	0
III.	,,	0.98	9.00	0.44	0
I.	20 mg. chloretone fed orally	1.05	4.08	0.43	0
II.	,,	1.37	4.95	0.36	0
III.	,,	0.93	4.08	0.44	0

TABLE V

*Mg. of ascorbic acid excreted per rat for a period of 4 days
before and after administration of the following substances.*

No.	Nature and quantity of the material.	No. of cases.	Free ascorbic acid.		Combined ascorbic acid.	
			Before.	After.	Before.	After.
I.	Chloralhydrate (100 mg.) in water (0.1 cc.)	1	0.56	0.45	0 49	0.38
		2	0.61	0.49	0.32	0.30
		3	0.60	0.42	0.45	0 20
II.	(a) Camphor (100 mg.) in coconut oil (0.2 cc.)	1	0.49	0.49	0.18	0.31
		2	0.61	0.84	0.27	0.29
		3	0.73	0.80	0.30	0.44
	(b) Camphor (50 mg.) in coconut oil (0.1 cc.)	1	0.19	0.26	0 75	0.96
		2	0.35	0.38	0.70	1.05
III.	Borneol (100 mg.) in alcohol (0.1 cc.)	1	0.32	0 45	0.84	0 60
		2	0.23	0.45	0.56	0.60
		3	0.35	0.53	0.61	0.70
		4	0.40	0.62	0.50	0.51
IV.	Menthol (100 mg.) in alcohol (0.1 cc.)	1	0.47	0.70	0.33	0.23
		2	0.42	0.67	0.38	0.38
		3	0.62	0.74	0.44	0.38
V.	Nicotine (10 mg.) in water (0.1 cc.)	1	0.70	0.50	0.31	0.30
		2	0.59	0.32	0.37	0.48
		3	0.52	0.45	0.21	0.53
VI.	Phenol (10 mg.) neutralized with NaOH (0.1 cc.)	1	0.47	0.92	0.27	0.77
		2	0.54	0.58	0.32	0.57
		3	0.64	0.73	0.29	0.36

SUMMARY

The administration of certain narcotics increased the output of free ascorbic acid in the urine of the rat and to a lesser extent in the urine of the guinea pig. The effects of chloretone, paraldehyde, phenobarbital, amidopyrine, urethane and bromobenzene have been studied.

A general depletion of tissue ascorbic acid especially of the organs like adrenal, liver and kidney, was observed in the narcotized guinea pig in most cases. The organs of rats on the other hand, showed increase in the concentration of ascorbic acid under similar narcotized conditions. This difference

is apparently connected with the fact that the rat has normally got the power to synthesise ascirbic acid which the guinea pig lacks.

The effect of feeding chloretone to the rat caused 400 per cent. increase in the excretion of free ascorbic acid with complete disappearance of combined ascorbic acid and at the same time the increased concentration of tissue ascorbic acid prove beyond doubt the increased synthesis of ascorbic acid by the rat in this condition. Knowledge about the mechanism of the synthesis is at present lacking.

The existence of combined ascorbic acid in the urine of the rat both under normal condition and under the influence of some organic compounds has been established by us using the method of Sen-Gupta and Guha for the estimations concerned.

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THE EFFECT OF ADMINISTRATION OF THYROID, 2:4-DINITROPHENOL AND INSULIN ON ASCORBIC ACID METABOLISM.

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During fever and other infections the excretion of free ascorbic acid in the urine is appreciably decreased (1) and larger quantities of ascorbic acid are required to be administered in order to maintain the normal excretory level. Similar decrease in the urinary excretion of both free and combined ascorbic acid due to the injection of diphtheria and tetanus toxins has been observed along with the depletion of ascorbic acid concentration in tissues like liver, kidney and adrenal (2-3). A marked diminution in the concentration of vitamin C in the adrenal glands of guinea pigs was also reported in cases of injection with (a) diphtheria, (b) pasturella, (c) mouse typhoid, (d) tuberculosis etc. (4). This diminution of ascorbic acid content in the urine as well as in the organs suggested the possibility of ascorbic acid being oxidised in the animal system due to increased metabolism. Experiments have been carried out by Ecklen and Kooy (5) on the artificial production of increased metabolism in the rats which were fatigued with forced work. This showed a decrease in the concentration of ascorbic acid in the organs. Various investigators (6) have fed desiccated thyroid as a means of increasing the metabolic rate and have shown diminution in the concentration of tissue-ascorbic acid in such experimental animals. The normally protective amount of ascorbic acid necessary to prevent scurvy in guinea pigs was found to be insufficient when administered with desiccated thyroid (7). Thaddea and Runne (8) have observed that the injection of thyroxin or thyrotropic hormone decreased the ascorbic acid content of the liver and adrenal of the

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guinea pig, while the removal of the thyroid gland increased the ascorbic acid concentration in those organs. Even artificially produced fever by keeping guinea pigs in a chamber maintained at temperature 100-106°F. showed depletion of tissue ascorbic acid, comparable with that of guinea pigs kept on a scorbutic diet for 15 days (9). The present investigation was carried out to throw further light on the problem and on the behaviour of combined ascorbic acid in the urine under such conditions of increased metabolism. The guinea pigs were fed with desiccated thyroid and with 2:4-dinitrophenol which are effective in increasing the metabolism. The effect of injection of 2 units of insulin in guinea pigs on the urinary excretion of both free and combined ascorbic acid has also been studied.

EXPERIMENTAL

The experimental procedure was the same as described previously (2-3). The urine of each guinea pig was collected and the quantities of ascorbic acid (both free and combined) excreted were estimated for a period of 3 consecutive days before and after the administration of the test substances. The test materials were (a) desiccated thyroid—1 tablet (0648 g.) per day, (b) 2 mg. of 2-4-dinitrophenol as its sodium salt and (c) 2 units of insulin (Lilly & Co.). The results concerning the urine are given in Table I.

Table II records the amounts of ascorbic acid present in the tissues of these experimentally treated animals.

TABLE I

Urinary excretion of free and combined ascorbic acid.

No. of Expt.	Nature of substances administered.	Mean value of ascorbic acid excreted per animal per day expressed in mg.			
		Free ascorbic acid.		Combined ascorbic acid.	
		Before administration.	After administration.	Before administration.	After administration.
I.	Thyroid (1 tablet) (0.0648 g.)	0.271	0.187	0.123	0.208
II.	,,	.164	.135	.123	.201
II.	,,	.164	.135	.123	.201
III.	,,	.238	.174	.114	.232
IV.	,,	.269	.112	.182	.212
V.	2:4-Dinitrophenol (2 mg.)	.157	.109	.224	.243
VI.	,,	.207	.124	.156	.233
VII.	,,	.207	.185	.163	.205
VIII.	Insulin (2 units)	.211	.244	.078	.083
IX.	,,	.267	.316	.047	.055
X.	,,	.198	.216	.126	.137

TABLE II

Tissue ascorbic acid.

No.	Substances administered	Mg. of ascorbic acid per g. of fresh tissue (mean value of 6 cases).		
		Liver.	Kidney.	Adrenal.
I.	Thyroid	0.130	0.069	0.249
II.	2:4-Dinitrophenol	.114	.062	.197
III.	Insulin	.127	.063	.201
IV.	Control	.177	.088	.346

DISCUSSION

It is evident from the observations of various investigators that the metabolic conditions influence the excretion of ascorbic acid in the urine. A smaller amount of ascorbic acid is excreted during fever and other infections *e.g.* pneumonia, tuberculosis, etc. Administration of thyroid and 2:4-dinitrophenol also diminished the excretion of free ascorbic acid in the urine. The combined ascorbic acid excretion was increased to a small extent. The depletion of tissue ascorbic acid was severe. Increased metabolism caused the disappearance of free ascorbic acid from the urine as well as from the tissues which cannot be accounted for by the amount of combined ascorbic acid excreted in the urine. The fate of the disappearing ascorbic acid is worthy of study. Incidentally it may be remembered that even under normal conditions only a part of ingested ascorbic acid is excreted in the urine. When repeated high doses of ascorbic acid are administered to guinea pigs so as to saturate the body completely, only 50 per cent. of the last administered ascorbic acid is excreted in the urine (10). In saturated conditions, the combined ascorbic acid excretion is totally stopped. The above results indicate that ascorbic acid is necessary for the upkeep of the normal metabolism of the body. Increase in the metabolic rate utilizes larger amounts of ascorbic acid. Narcotics and anesthetics on the other hand increase the ascorbic acid excretion in certain cases but in many cases the ascorbic acid content of the tissues was found to be decreased (11).

Insulin injection caused slight increase in the excretion of both free and combined ascorbic acid in the urine, while the tissues suffered marked depletion.

SUMMARY

The urinary excretion of free ascorbic acid decreased when guinea pigs were fed with 1 tablet (0.0648 g.) of desiccated thyroid and also when fed with 2 mg. of 2:4-dinitrophenol. The excretion of combined ascorbic acid in both cases increased. Injection of 2 units of insulin produced very slight increase in both free and combined ascorbic acids.

The ascorbic acid content of the tissues especially liver, kidney and adrenal has been compared with that of the untreated controls.. A decrease in the concentration of of ascorbic acid in all the tissues was observed when the guinea pigs were administered with thyroid, dinitrophenol and insulin. Increased metabolism, artificially induced, thus appears to use up ascorbic acid, part of which is excreted as combined ascorbic acid but the larger part of which is apparently metabolised.

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STUDIES ON THE ACTIVITY OF BLOOD SERUM ESTERASE UNDER DIFFERENT CONDITIONS OF VITAMIN C-NUTRITION.

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Pantschenko-Jurewicz and Krant (1) claimed to have identified ascorbic acid as a coferment in the esterase preparation of the liver and supposed that the esterase activity was due to the catalytic function of ascorbic acid present in such preparation. Kretesz (2) and others (3) were unable to confirm the presence of ascorbic acid in the esterase preparation of the pig liver. Only recently Goldstein and Bondareva (4) have reported the inactivation of esterase preparation of the liver when incubated with ascorbic acid oxidase. The inactivation of esterase by the oxidase does not appear to be sufficient to prove the presence of ascorbic acid in the liver esterase.

From time to time reports have been made about the existence of a possible relationship between esterase activity and the concentration of ascorbic -acid in the tissues like liver, kidney, intestine and blood of the guinea pig. Raabe (5) was, however, unable to establish a constant relationship between ascorbic acid content and esterase activity in the liver, kidney, intestine and blood of normal guinea pigs. Only intravenous injection of ascorbic acid in the guinea pig caused a temporary rise in the esterase activity in

the liver.   Harren and King (6) have recently observed a progressive decrease in the esterase activity in the liver of guinea pigs along with the severity of scurvy.   In the present communication attempts have been made to study the esterase activity of blood serum under different conditions of vitamin C intake namely, during the normal, scorbutic and vitamin C-saturated conditions.   *In vitro* experiments, with the addition of ascorbic acid, on the esterase activity of the serum of normal guinea pigs have been carried out in order to investigate how far ascorbic acid is responsible for the catalytic hydrolysis of ethylbutyrate.

EXPERIMENTAL

Male healthy guinea pigs, weighing between 200 and 300 g., were selected for the purpose.   The animals were divided into seven groups.   The first three groups were fed on germinated gram and green grass, while the last four groups were kept on a scorbutic diet which consisted of crushed oats 65 parts, casein 12 parts, crushed gram 20 parts, calcium carbonate 3 parts, common salt 1 part and 2 cc. of cod-liver oil per 100 g. of the above diet. The blood was drawn out from the heart of the guinea pig and was transferred into a clean centrifuge tube and was allowed to clot.   The clotted blood was centrifuged.   The serum which separated from the clotted blood was taken out in a dry test tube by means of a pipette fitted with a rubber cap. 0.25 Cc. of serum was employed for each determination of the esterase activity.

The esterase activity was determined in the following manner (7).   In a 100 cc. Erlenmeyer flask, 20 cc. of M/80 phosphate buffer of pH 8.0, 0.25 cc. of phenolphthalein solution (1%) and 0.25 cc. of ethylbutyrate were taken, to which 0.25 cc. of serum was added.   The flask was then well corked and kept for two hours at 37° in a thermostat without stirring.   After 2 hours the flask was cooled under tap water and the contents of the flask were immediately titrated against N/100 NaOH.   Controls containing every substance except serum were similarly titrated.   The difference in the consumption of NaOH between the control and the experimental flask gave the measure of the esterase activity in the serum of the guinea pig under different experimental conditions.   Each set of results was checked by duplicate experiments.   The results are given in Table I.   Controlled experiments, with the addition of 0.1 mg., 0.05 mg., 0.025 mg. of pure ascorbic acid *in vitro*, on the hydrolysis of ethylbutyrate both in presence and in absence of blood serum of normal guinea pigs, indicate that ascorbic acid takes part neither in the catalytic activity of the esterase nor in the hydrolysis of ethylbutyrate.   The method of determination in these cases was the same as described above.

TABLE I

No. of Expt.	Condition of the experimental animals.	Animal No.	Esterase activity expressed in cc. of N/100 NaOH consumed.
I.	Guinea pigs fed on green grass and germinated gram.	1	2.0
		2	2.6
		3	3.4
		4	3.9
		5	3.2
		6	3.3
II.	Guinea pigs fed on the same diet for 21 days.	1	3.1
		2	3.0
		3	3.6
		4	2.7
		5	3.5
		6	3.3
III.	Guinea pigs kept on the same died supplemented with 50 mg. of ascorbic acid daily for a period of 7 days.	1	3.7
		2	3.7
		3	3.8
		4	3.8
		5	3.8
		6	3.8
IV.	Guinea pigs kept on scorbutic diet for 7 days.	1	3.2
		2	3.3
		3	3.1
		4	3.1
.V.	Guinea pigs kept on scorbutic diet for 14 days.	1	3.1
		2	3.6
		3	3.2
		4	2.6
		5	1.8
VI.	Guinea pigs kept on scorbutic diet for 21 days.	1	2.1
		2	2.7
		3	2.1
		4	1.2
		5	1.1
		6	1.1
		7	1.2
		8	1.3
VII.	Guinea pigs kept on scorbutic diet for 28 days supplemented with 5 mg. of ascorbic acid for first 7 days of scurvy.	1	1.7
		2	3.4
		3	3.7
		4	2.4
		5	3.0
		6	2.7

DISCUSSION

It is evident from Table I that there is a wide range of individual variations in the activity of the serum esterase of the guinea pigs kepts on a normal diet which consisted of green grass and germinated gram. Uniform results were, however, obtained when the animals were kept for a long period on the same 'diet. Higher serum esterase activity can be observed in the animals which were daily fed with 50 mg. of pure synthetic ascorbic acid for a period of seven days. It has been observed in this laboratory that such administration of 50 mg. of ascorbic acid is sufficient to saturate guinea pigs with vitamin C. In this kind of nutritional experiments it is desirable to compare one set of results obtained with animals kept on normal diet with those obtained with animals saturated with ascorbic acid. The esterase activity in the saturated condition becomes more uniform. The esterase activity decreases slowly during first fourteen days and after 21 days of scurvy the esterase activity suddenly drops considerably. The results in Table I indicate that ascorbic acid may not be a co-ferment in the esterase present in the serum but may, by its presence, stimulate the production of esterase activity by some unknown mechanism in the animal system.

SUMMARY

In so-called normal guinea pigs there was wide individual variation in the serum esterase activity depending on the degree of saturation of the body with ascorbic acid. In saturated conditions, *i.e.* by feeding normal guinea pigs with 50 mg. of ascorbic acid continually for 7 days, the serum esterase activity increased appreciably and more uniform results were obtained.

When ascorbic acid was withdrawn from the food the esterase activity decreased and after 21 days of scurvy the esterase activity decreased appreciably.

Addition of synthetic pure l-ascorbic acid *in vitro* neither increased the esterase activity of the serum of the normal animal nor helped in the hydrolysis of ethylbutyrate.

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ANTIGENIC PROPERTIES OF CRYSTALLINE
HÆMOLYSIN

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Enzymes in several cases have been proved to be antigenic. Enzymes like urease, pepsin and pepsinogen, chymotrypsin and chymotrypsinogen have been used as antigens. Sumner (1) obtained antiurease from urease and described its properties with sufficient exactness. Northrop (2) using swine pepsin obtained pepsin precipitating antibodies. As pepsin is inactivated above pH 6.0, the non-existence of active pepsin is beyond doubt in the body fluids. So the antibody produced is of the inactive denatured pepsin. Seastane and Herriot (3) have demonstrated that alkali denatured pepsins from swine, cattle and guinea-pigs form precipitate with swine antiserum, but alkali denatured pepsins from rabbit, chicken and shark do not behave in the same way.

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Tira (4) obtained anticatalase from the different varieties of crystalline catalase. By means of the anaphylactic test with Dale technique Ten Broeck (5) was able to differentiate pig and beef trypsin as well as chymotrypsin.

As crystalline hæmolysin has been prepared from the venom of some snakes, it is worth while studying their specificity immunologically. The effects of ultraviolet light and of oxidation on crystalline hæmolysin were also investigated to secure information as to the immunological nature of the enzyme. The production of immunity with reversibly inactive enzymes and not with irreversibly inactive enzymes is of great importance because from it some insight into the antigenic character and also to the chemical nature of these agents may be obtained.

Crystalline hæmolysin has also been subjected to oxidation and reversible inactivation where the presence of sulphydryl group has been postulated in all cases and the results will be published later. Pincussen (6) showed that urease was inactivated by ultraviolet light but boiled urease has a reactivating effect on it. Tauber (7) found that ultraviolet light inactivates crystalline urease and the inactivation was found to be inversely proportional to the distance of the sample from the arc. Kubowitz and Haas (8) observed that the band most absorbed is the most effective in causing destruction.

EXPERIMENTAL

Crystalline hæmolysin

Crystalline hæmolysin has been obtained from three varieties of venoms. A short note on the production of crytalline hæmolysin has been published (9). A detailed description of the method of preparing the three crystalline products will be published later. The activities of the crystalline hæmolysins obtained from monocellate and binocellate varieties of *Naja Tripudians* venom are 3360 and 3318 hæmolytic units per mg. of the crystalline products, while the activity of the crystalline hæmolysin· obtained from *Bungarus Fasciatus* venom is 1650 hæmolytic units per mg. of the product.

Production of Antihæmolysin

Two rabbits were given injections at intervals of 3 days during a period of 30 days, the dose used being 5.0 mg. of hæmolysin obtained from monocellate variety of *Naja Tripudians* venoms. Both rabbits were found to be well immunised at the end of the 30 days period. The antihæmolytic activity was high in both the rabbits. The production of antihæmolysin was tested by its ability to precipitate hæmolysin.

The following method was used. 5 cc. of blood were taken from an ear vein of the rabbit and allowed to clot normally. in a 10 cc. centrifuge tube. The clot was broken up and centrifuged to separate the serum. To test tubes containing 1 cc. of serum or serum diluted with 0.85% NaCl, 1 cc. of hæmolysin at various dilutions and 8 cc. of 0.85% NaCl were added. The tubes were kept at 37° for 30 minutes and were then examined. It was found that when the dilution of serum was 1:10, 10 cc. of this diluted serum produced maximum precipitation with 1.5 mg. of the hæmolysin.

TABLE I

Precipitin reaction.

Crystalline hæmolysin 1.5 mg. total voume 10 cc.		Anti-hæmolysin 1 cc. total volume 10 cc.	
Serum.	Degree of precipitation.	Hæmolysin.	Degree of precipitation.
1.0 cc.	+ + +	15.0 mg.	+
0.1	+ +	1.5	+ + +
0.01	+ +	0.15	+ +
0.001	+	0.015	+
0.0001	—	0.0015	—

Differentiation of hæmolysins from venoms of monocellate and binocellate variety of "Naja Tripudians" and "Bungarus Fasciatus" venom by means of precipitin reaction.

Three rabbits were immunised for a period of 30 days against crystalline hæmolysins obtained from Fasciatus venom and from the two variety of *Naja Tripudians* venom. These dose used was the same as used in the previous case. The maximum amount of blood was taken out from the heart of the animals and allowed to clot normally and the serum separated. The cross-precipitation tests were carried out with the different antisera and the different hæmolysins. For this purpose, to 0.2 cc. of immune serum were added different amounts of hæmolysin of each variety. The total volume made up to 2 cc. with 0.85% NaCl and incubated for 30. minutes at 37° and the formation of precipitate or turbidity noticed in each case.

TABLE II

(*a*) Tests with 0.2 cc. immune serum produced by using monocellate variety of *Naja Tripudians* hæmolysin as antigen.

Hæmolysin.	Antigens used.		
	Naja Tripudians (var monocellate) hæmolysin.	*Naja Tripudians* (var binocellate) hæmolysin.	*B. Fasciatus* hæmolysin.
2.0 mg.	—	—	—
0.2	+ +	+ +	—
0.02	+ + +	+ + +	+ +
0.002	+ +	+ +	—

(*b*) Using immune serum prepared from binocellate variety of *Naja Tripudians* hæmolysin exactly similar behaviour was observed with the three different varieties of the hæmolysins.

TABLE III

(*c*) With 0.2 cc. immune serum prepared by using *B. Fasciatus* hæmolysin as antigen.

Hæmolysin.	Antigens used.		
	Naja Tripudians (var monocellate) hæmolysin.	*Naja Tripudians* (var binocellate) hæmolysin.	*B. Fasciatus* hæmolysin.
2.0 mg.	—	—	—
0.2	+	+	+ +
0.02	+ +	+ +	+ + +
0.002	±	±	+ +

From the above experiments it can be concluded that hæmolysins in the venoms of monocellate and binocellate varieties of Naja Tripudians are similar in nature while the hæmolysin in B. Fasciatus venom is of different nature as judged from its cross-precipitation reaction with the antisera prepared from the hæmolysins isolated from the two varieties of *Naja Tripudians.*

Determination of the antihæmolytic activity of the different sera was carried out by adopting the following method: 1 Cc. of immune serum was added to a test tube containing 1.5 mg. of hæmolysin. The total volume was made up to 10 cc. by the addition of 0.85% NaCl. The tube was incubated at 37° for 30 minutes and the content was then centrifuged. The lecithinase (hæmolytic) activity was determined from 1 cc. of 100-fold diluted supernatant liquid by the method described by the author (10). The experiment was repeated with the serum of normal rabbit, to which has been added the same amount of hæmolysin and which had been diluted in the same way. Thus the units of lecithinase (units of hæmolysin) precipitated by the anti-hæmolysin in 1 cc. of the immune serum was determined.

TABLE IV

Antihæmolytic activity of different sera.

One cc. of antiserum against	Amount of different hæmolysins neutralised.		
	Naja Tripudians (var monocellate).	*Naja Tripudians* (var binocellate).	B. Fasciatus.
Naja Tripudians hæmolysin (var monocellate)	0.26 mg.	0.27 mg.	0.12 mg.
Naja Tripudians hæmolysin (var binocellate)	0.28	0.28	0.13
B. Fasciatus hæmolysis	0.115	0.12	0.22

It is observed here that 1 cc. of antisera from each of the monocellate and the binocellate variety of *Naja Tripudians* hæmolysin neutralises equivalent amount of either of the two hæmolysins, but the amount B. Fasciatus hæmolysin neutralised is less than half the amount of the other two hæmolysins neutralised. The antisera prepared from B. Fasciatus hæmolysin neutralises the hæmolysins from Naja Tripudians venom but the amount neutralised in each case is less than one half of its homologous antigen.

Effect of ultraviolet light and of oxidation on crystalline hæmolysin in relation to its immunological behaviour.

It has been shown that crystalline hæmolysin can be reversibly inactivated by oxidising agents like iodine, hydrogen peroxide and ferricyanide. After controlled inactivation the inactivated product was reactivated with hydrogen sulphide and potassium cyanide.

The effect of ultraviolet light on crystalline hæmolysin and the effect of reducing agents like hydrogen sulphide and potassium cyanide on the irradiated product was studied. Irradiation was carried out by a mercury vapour lamp of Heraus type at a distance of 30 cm. Samples were taken at intervals of 5, 10, 15, 20, 30, 60 minutes. It was found that within 10 minutes 1% solution of crystalline hæmolysin was half inactivated by irradiation. The irradiated product was immediately treated with the above mentioned reducing agents but no reactivating effect was noticed.

Immunization was then carried out with crystalline hæmolysin and its irradiated and oxidised products. The irradiated and oxidised hæmolysins used for immunization have lost 95% of their original activity. It was found that immunity was produced by the oxidised hæmolysin and the crystalline untreated hæmolysin but the irradiated hæmolysin but the irradiated hæmolysin failed to produce a precipitate with the serum obtained from rabbits immunized with it. Cross-precipitation tests of the various enzyme preparations and their antisera were carried out as before.

TABLE V

*Cross-precipitation tests on different sera
and hæmolysin preparations.*

Antiserum from	Antigen used.		
	Irradiated hæmolysin.	Oxidised hæmolysin.	Active hæmolysin.
Oxidised hæmolysin	+ +	+ +	+ + +
Irradiated hæmolysin	+ + +	+	—
Crystalline hæmolysin	+ +	—	+ + +

DISCUSSION

It has been shown that oxidised hæmolysin produces antihæmolysin almost in a proportion equivalent to that obtained by the use of the active enzyme but the irradiated enzyme loses this property. It has also been shown that the oxidised enzyme can be reactivated but the irradiated enzyme cannot be reactivated. The change that takes place in the oxidised enzyme is either in the side chain or in some chemical linkage in the compound. The most probable assumption is that the -SH groupings are oxidised to -S-S- groupings and on injection into animals it is converted to SH grouping by the reducing substances present in the tissue fluid, so that the antibody produced is that of the active enzyme. The results recorded in Table V can be explained on the assumption that the active hæmolysin consists of a portion (S) containing the determinant group and a protein portion (P) which are chemically linked together. On oxidation only S is affected and changed to S' say, while on irradiation S remains practically unaffected but P is altered to P' say and the link between S and P is broken. This can be represented in the following way:

$$S + P' \quad \xleftarrow{\text{irradiation}} \quad S-P \quad \underset{\text{reduction}}{\overset{\text{oxidation}}{\rightleftarrows}} \quad S'-P$$

<div align="center">Active enzyme</div>
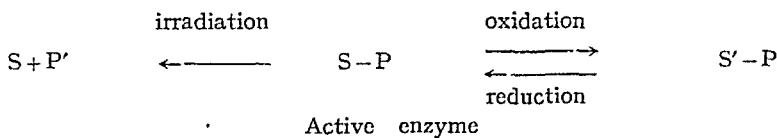
Therefore on the basis of Landsteiner's (11) theory one would expect that when the irradiated enzyme is injected into an animal the antibody produced is against P' and hence it reacts strongly with P' and very weakly if at all with the antigens S-P or S'-P. On the other hand the antibody produced by the injection of the untreated crystalline hæmolysin (S-P) can react fairly strongly with the irradiated enzyme as it contains the portion S which carries the determinant group unaltered.

SUMMARY

1. Crystalline hæmolysin is irreversibly destroyed by short exposure to ultraviolet light.

2. The pricipitin reaction indicates that hæmolysin from the two varieties of Naja Tripudians venom are more closely related to each other than they are to the hæmolysin isolated from Bungarus Fasciatus venom.

3. Sulphydryl groupings may enter into the antigenic specificity of the enzyme.

4. Oxidised hæmolysin produces antibody against active crystalliñe hæmolysin' but irradiated hæmolysin fails to produce such an antibody.

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SOME OBSERVATIONS ON THE COMPLEMENT IN THE SERUM OF MONKEYS DURING INFECTION WITH *PLASMODIUM KNOWLESI*

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While titrating sera of monkeys for complement fixing antibodies, it has been observed (1) that sera from monkeys with acute or subacute infection very frequently proved anticomplementary. On the basis of such observation it was decided to estimate the amounts of complement in the sera of monkeys during various phases of *P. knowlesi* infection as compared with normal or non-infected monkeys.

EXPERIMENTAL

1. *Monkey Serum :*—Samples of blood were collected from the femoral vein or heart of monkeys in the morning in empty stomach. When it

clotted, serum was separated, freed from R.B.C. by centrifuging and kept in ice box.` Complement titration test was performed on the same day with fresh serum.

2. *Amboceptor :*—Anti-sheep-R.B.C. hemolysin was obtained by immunising rabbits with washed red cells of sheep. After titration it was kept in ice box for future use. One unit of amboceptor was the smallest amount of diluted serum which would hemolyse 0·4 cc. of 5% suspension of sheep's R.B.C. in the presence of 0·05 cc. of fresh pooled guinea-pigs' serum in half an hour at 37°C.

3. *Red Blood Cells :*—5% emulsion of washed red blood cells of sheep was used. Owing to the low complement content of the monkeys' sera 0·2 cc. of the R.B.C. emulsion was used in each of the present series of experiments for the titration of complement in the monkey serum.

4. *Complement :*—Fresh guinea-pigs' serum was used as complement in titration of hemolysin and also in hemolytic control in complement titration in monkey serum.

While recording complement in the serum of monkeys, one unit of complement was taken as the minimum amount of 1 in 5 dilution of fresh serum, which would hemolyse 0·2 cc. of 5% emulsion of sheep's R.B.C. in the presence of one unit of amboceptor. The test proper was set up as follows:—

1 in 5 dilutions of monkey serum in different quantities in a series of tubes.

Amboceptor (diluted)	1 unit.
Sheep's R.B.C. (5% emulsion) ...	0·2 cc.
Saline	up to 2 cc.

Controls: 1. Saline control (without complement).
2. Hemolytic control—with one unit of amboceptor, one unit of complement as guinea-pigs' serum and 0·4 cc. of 5% sheep corpuscles.

Reading: Reading was taken after incubation of the mixture at 37°C for half an hour.
Monkeys used in the present series of experiments belonged to *Silenus Rhesus* group.

TABLE I.

Showing complement in the normal monkey serum.

Total number of normal monkeys' serum examined.	Minimum quantity of 1 in 5 dilution of serum containing 1 unit of complement.	Maximum quantity of 1 in 5 dilution of serum containing 1 unit of complement.	Average quantity of 1 in 5 dilution of serum containing 1 unit of complement.
10	0.1 cc.	0.4 cc.	0.2 cc.

It can be seen from the above table that while there was moderate variations in the normal complement titre in the monkeys' serum, the average titre was one unit in 0.2 cc. of 1 in 5 dilution of serum.

TABLE II.

Showing variations in the complement titre during the course of acute infection with P. knowlesi.

Serial number of monkeys.	Quantity of 1 in 5 dilution of sera before infection, containing 1 unit of complement.	Days of infection.	Parasite count in the peripheral blood showing the percentage of R.B.C. infected.	Quantity of 1 in 5 dilution of serum containing 1 unit of complement.
83	0.1 cc.	4th day (terminal phase)	49%	1.2 cc.
84	0.1	(a) 4th day (b) 9th ,, (terminal phase)	1 22	0.3 0.8
85	0.1	(a) 4th day (b) 8th ,, (terminal phase)	1 21	0.4 1.0
91	0.3	(a) 3rd day (b) 6th ,, (terminal phase)	0.2 25	0.4 0.8
93	0.2	(a) 8th day (b) 10th ,, (c) 12th ,, (terminal phase)	2 6 28	0.4 0.5 above 1.6

Table II shows that while there was some reduction of the complement in the earlier stages of infection, the fall in its titre in the terminal phase was precise and sharp. It may be noted here that monkeys, which received heavy infecting dose of *P. knowlesi* by intravenous route showed malaria parasites in the peripheral blood soon after injection and in most of the monkeys which were inoculated with small doses of parasites by subcutaneous injections, 4 to 6 days passed as incubation period after which parasites appeared in the peripheral blood. Then the parasite count increased rapidly and the monkey died in another 4 to 6 days. The monkeys in the above table included both the groups.

In the present series of experiment by terminal phase of acute infection is meant roughly the last 24 hours before death of the monkey, when the animal was very sick and usually above 20% of the red cells were infected with *P. knowlesi*.

TABLE III.

Showing complement in the serum of monkeys during the terminal phase of acute infection.

Total number of monkeys examined.	Minimum quantity of 1 in 5 dilution of serum containing 1 unit of complement.	Maximum quantity of 1 in 5 dilution of serum containing 1 unit of complement.	Average quantity of 1 in 5 dilution of serum containing 1 unit of complement.
10	· 0.6 cc.	Above 1.6 cc. (in one case there was no hæmolysis in 1.6 cc.).	1.04 cc.

While 0·2 cc. of 1 in 5 dilution of serum of average normal monkeys contained 1 unit of complement, the same amount of complement was present in 1·04 cc. of 1 in 5 dilution of serum of average monkey during the terminal phase of acute infection.

TABLE IV.

*Showing complement in the serum of monkeys with chronic
infection or super-infection with P. knowlesi.*

Group of monkey.	Total number of monkeys examined.	Duration of infection.	Minimum quantity of 1 in 5 dilution of serum containing 1 unit of complement.	Maximum quantity of 1 in 5 dilution of serum containing 1 unit of complement.	Average quantity of 1 in 5 dilution of serum containing 1 unit of complement.
Chronic infection	3	4-6 months	0.20 cc.	0.30 cc.	0.23 cc.
Super-infection	3	4-9 months	0.30 cc.	0.40 cc.	0.36 cc.

The number of monkeys belonging to the group of chronic infection and super-infection was small. While in the chronically infected monkeys the complement titre was similar to those in the normal monkeys, in the super-infected group the titre was slightly lower than normal. This may be due to the fact that after injection of first few super-infecting doses of infected blood, there occurred moderate parasitic relapses in the monkeys, which produced a temporary subacute infection. In the latter condition, it has already been shown, there appeared some fall in the titre of complement.

It can be seen in the above series of experiments that, while in average normal or non-infected monkeys one unit of complement was present in 0·20 cc. of serum, in the group of monkeys with acute infection with *P. knowlesi* one unit of complement was present in 1·04 cc. of serum, when it was collected during the terminal phase of acute infection. It was also noted that after injection of an infecting dose of *P. knowlesi* in monkey there was no appreciable fall in the titre of complement during the incubation period (when the peripheral blood remained free from parasites) and during the earlier part of infection, when the peripheral blood showed comparatively low percentage of infected R.B.C. But during the last 3 or 4 days of acute infection there was progressive loss of complement in the blood. During the terminal phase of acute infection there appeared sharp fall in the complement content of the serum. When monkeys were treated in the latter part of acute infection the fall of complement in the serum of blood of these treated monkeys was arrested and gradually came to normal titre *paripassu* with the fall in the parasite count.

It has been observed that there is a fall in the complement titre in the serum of human beings as well as in experimental animals during many of the acute bacterial infections (2). The sharp nature, by which the titre of complement falls in the serum of monkeys during the terminal phase of acute infection with *P. knowlesi* is very instructive. Whether the loss of complement is due to biochemical changes in the blood as a result of destruction of large number of R.B.C. in the process of Schizogony of the plasmodia or some toxin liberated by the huge number of malaria parasites or it is due to the complement being utilised by the defensive mechanism of the host in an attempt to combat the parasites or there are some other factors responsible, has not been investigated in the present series of experiments.

SUMMARY

Sharp fall in the complement in the serum of monkeys during the latter part of acute infection with *P. knowlesi* has been observed.

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INDEX OF SUBJECTS

EFFECT OF ALKALOIDS ON THE ABSORPTION OF MONOSACCHARIDES FROM THE GUINEA-PIG INTESTINE. PART I. EFFECT OF ATROPINE, STRYCHNINE AND QUININE ON THE ABSORPTION OF GLUCOSE, FRUCTOSE, XYLOSE AND ARABINOSE

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It has been demonstrated by several workers (1 7) that of all the monosaccharides, galactose, glucose and to a certain extent, fructose, are preferentially absorbed from the intestine and that some specific activities of the epithelial cells of the villi are responsible for this phenomenon. According to Verzar (5-7), these sugars after their diffusion into the mucosal cells are selectively phosphorylated. The constant transformation of these sugars inside the intestinal mucosa maintains the diffusion gradient at a high level and thus affects the velocity of absorption.

This paper deals with the effects of atropine, strychnine and quinine on the absorption of glucose, fructose, xylose and arabinose from the guinea-pig intestine.

EXPERIMENTAL

Normal male guinea-pigs were used and these were kept without food for 48 hours before the beginning of the actual experiments ; water was however given *ad libitum*. 1.8 Mg. of urethane per g. of body-weight was injected intramuscularly. After $2\frac{1}{2}$-3 hours following the injection, when the animal was completely anæsthetised, the abdominal wall was opened and two loops (15-20 cm.), one at the duodenal and the second at the ileac end, were carefully ligatured with silk thread, keeping the circulation intact as far as possible. The gut was never washed because the small intestine was found to be completely free from food residues after 48 hours fasting (8). To allow the animal to recover from the shock of the operation it was kept at 37° for one hour. Different strengths of quinine, atropine and strychnine were prepared by using isotonic sugar solutions, 5.4% hexoses and 4.5% pentoses. The effect of the drugs was always compared with the rate

of absorption of aqueous sugar solutions in the same animal. 1.5 Cc. of the test solutions were introduced gently into the loops by using a fine hypodermic needle. After 1 hour the animals were sacrificed and the intestinal contents were separately washed with ice cold water, the washings being subsequently brought to volume with distilled water in 100 cc. volumetric flasks. The intestinal mucosa was then scraped out—the scrapings were weighed accurately and extracted with 5 cc. of 10% ice-cold trichloracetic acid. This was filtered and definite volumes of the acid filtrate were taken to estimate the inorganic and total-acid soluble phosphates by Fiske and Subbarow's method (9). The sugars were estimated by Hagedorn and Jansen's method (10). Fructose, xylose and arabinose values were obtained from a calibrated curve.

The findings are summarised in Tables I and II.

TABLE I

The figures express mg. of sugar absorbed per metre of intestine per hour.

| Sugars | Relative rates of absorption of sugar solutions, with and without alkaloids | | | | From | | | Remarks |
	Atropine 0.1 g. %	Strychnine 2.2 mg. %	Quinine 0.2 g. %	Normal	Duodenum	Ileum		
1.5 cc. of 5.4% glucose	... 170	230	322	230	206	260		36% (−) due to atropine. 4.2% (+) due to strychnine. 40% (+) due to quinine.
1.5 cc. of 5.4% fructose	... 136	205	203	202	—	—		33% (−) due to atropine. Strychnine—no effect. Quinine—no effect.
1.5 cc. of 4.5% xylose	... 121	161	157	158	159	157		4% (+) due to strychnine. 31% (−) due to atropine. Quinine—no effect.
1.5 cc. of 4.5% arabinose	... 106	140	138	136	—	—		28% (−) due to atropine. Quinine—no effect. Strychnine—no effect.

TABLE II

Phosphate fractions in trichloracetic acid extract of the intestinal mucosa. The figures express mg. of phosphate present in 100 g. of intestinal mucosa (wet).

Experiment	Total Phos.	Inorg. Phos.	Org. Phos.	Org. Phos. / Total Phos.	Percentage of variation of Org. Phos. / Total Phos.
Glucose	171	119	52	0.301	
Quinine+ Glucose	160	103	57	0.352	17.2% increase.
Atropine+ Glucose	156	120	36	0.230	23.6% decrease.
Strychnine+ Glucose	158	99	59	0.315	4.6% increase.
Fructose	112	33	79	0.706	
Quinine+ Fructose	110	33	77	0.696	1.1% decrease.
Atropine+ Fructose	100	42	58	0.580	18% decrease.
Strychnine+ Fructose	108	36.5	71.5	0.709	1.0% increase.

DISCUSSION

It will be seen from Table I that the rate of absorption of the different sugars is profoundly influenced by the alkaloids.

Atropine sulphate used in a concentration of 0.1% decreases the rate of absorption of all the sugars and the rate of decrease is as follows:

Glucose	...	...	...	... 36%
Fructose	...	...	...	... 33%
Xylose	...	...	...	... 31%
Arabinose	...	...	...	... 28%

Strychnine used in a concentration of 2.2 mg.% has no action on the mechanism of absorption but quinine sulphate (0.2%), though it does not affect the rate of fructose, xylose and arabinose, increases glucose absorption by 40%.

To elucidate the precise nature of the effects produced by these alkaloids on intestinal absorption, the following points were studied:—

(1) The organic phosphorus content in the intestinal mucosa after the sugars were allowed to be absorbed for one hour. (2) The relative rates of diffusion of

an hexose and a pentose, through a living animal membrane, with and without alkaloids. (3) The individual effects of the different sugar solutions with quinine on the tone and motility of the intestine.

There is a decrease of 23.6% and 18% in $\dfrac{\text{organic phosphate}}{\text{total phospate}}$ ratio due to the presence of atropine in glucose and fructose solutions during absorption. Strychnine does not influence the ratio, while quinine increases the ratio by 17% when used with glucose but the ratio is not altered when it is used with fructose. (Table II).

The rates of diffusion of glucose and xylose through an animal membrane are not at all influenced by atropine, strychnine and quinine (Table III).

TABLE III

The relative rates of diffusion of sugar solutions, with and without alkaloids, through goat's peritoneum. 1 Cc. of glucose or xylose solution was allowed to diffuse for 30 mins. against 150 cc. of distilled water. The figures express mg. of sugar diffused out.

Sugar.	Normal.	With quinine 0.2 g. %	With atropine 0.1 g. %	With strychnine 2.2 mg. %	Remarks.
Glucose (5.4%)	29	29	29	29	Quinine, atropine and strychnine do not affect the diffusion rate of glucose and xylose.
Xylose (4.5%)	34	34	34	34	

The effects of the above alkaloids on the tone and motility of the intestine are very well known. Atropine sulphate causes a depression of both tone and motility (11-13). Strychnine, given in small or even tetanic doses, does not affect the movement or tone of the gastro-intestinal tract (14), while quinine increases them (15).

The effect of quinine on the rate of absorption of the different sugars is however peculiar. Though this alkaloid increases the rate of absorption of glucose by 46%, it has got no effect on the rate of absorption of the other sugars. It is however expected from its effects mentioned above on the tone and motility of the intestine that if it increases the rate of absorption of any one sugar, it should increase the rate of others also. The effect of quinine dissolved in the different sugar solutions on the tone and motility of intestine was therefore recorded and to avoid the direct effect of this alkaloid on the outer wall of the intestine, the following apparatus, a diagrammatic representation of which is given (Fig. I), was used instead of using the usual Dale's apparatus. The position of reservoir was so adjusted that the peristaltic movement starts in the intestine. The reservoir

was fixed at that point. A hypodermic needle attached to a syringe carrying
1.5 cc. of the test solutions was introduced within the tube no. I, by puncturing
the rubber tubing ; the tip of the needle was brought as near as possible to the
bottom end of the intestine, and the test solution was slowly injected. It will be
noted from Figures I and II that whereas quinine in a concentration of 0.2% made
up in 5.4% glucose solution increases the tone of the intestine to a very significant
extent, this very same alkaloid used in the same concentration with the other sugars,
fails completely to alter the spontaneous tone exhibited by the intestine.
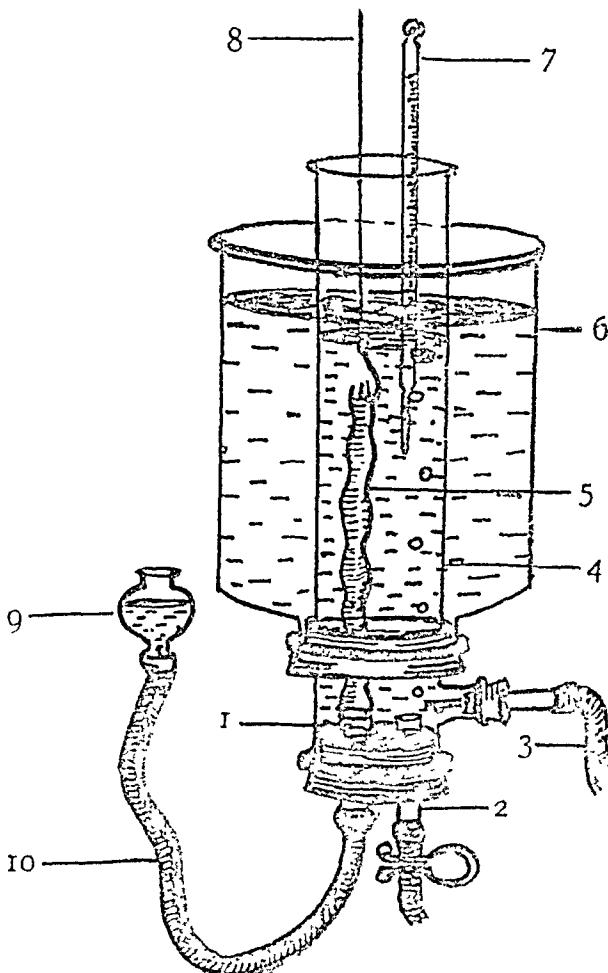
FIG. I.

(1) Tube with a constriction at the top to which the intestine was tied.
(2) Tube to drain out the contents of (1).
(3) Side tube to bubble in oxygen.
(4) Tube filled up with Dale's mammalian Ringer.
(5) Intestine—10 cm. in length.
(6) Glass jacket containing approximately 2 litres of water maintained at 37°C.
(7) Thermometre.
(8) String attached to the writing lever.
(9) Reservoir containing Dale's mammalian Ringer.
(10) India rubber tubing.

Fig. II
Effect of glucose and glucose + quinine.
G—Glucose. ˙Q—Quinine Glucose.
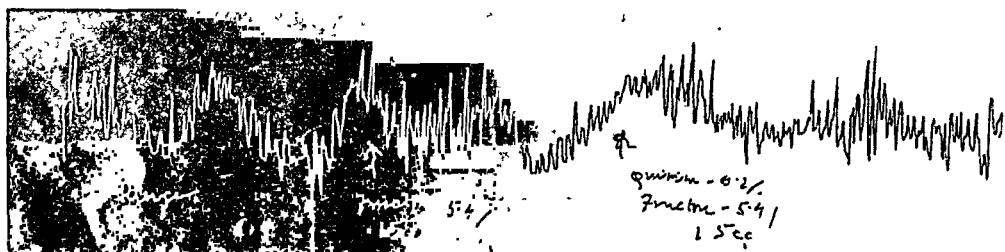
A
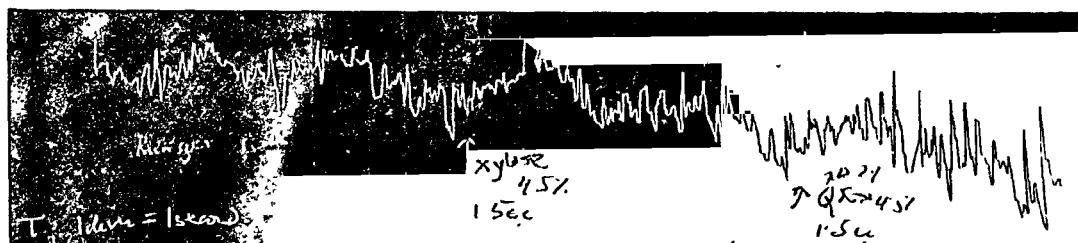
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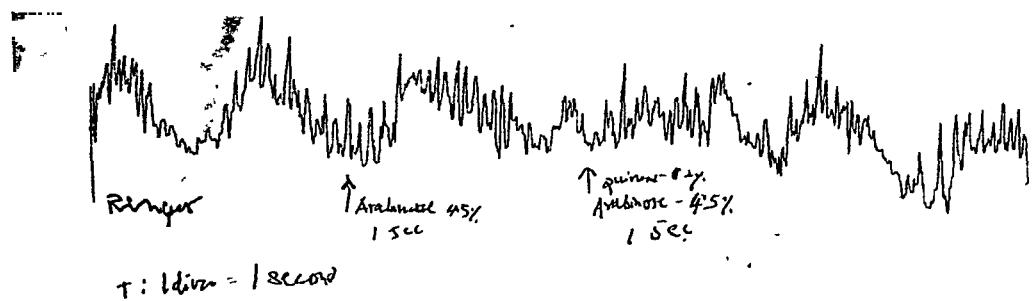
C

Fig. III
A. Effects of fructose and fructose + quinine.
B. Effects of xylose and xylose + quinine.
C. Effects of arabinose and arabinose + quinine.

From the foregoing discussion, it is concluded that (i) the increased rate of glucose absorption in presence of quinine is due not only to the increased rate of phosphorylation of this sugar but also due to increased tone of the intestine induced by this alkaloid ; (ii) the decreased rates of absorption of glucose and other sugars from solutions containing atropine is due to the decreased intestinal motility produced by atropine coupled with its effect on phosphorylation ; (iii) strychnine which has no effect either on the intestinal motility or phosphorylation, does not influence the rates of absorption of different sugars to any appreciable extent.

Lajos (16) reports that the blood sugar curve, after ingestion of glucose, is reduced by the administration of atropine, while Hughes (17) found that the blood sugar is raised by quinine. It appears from the present investigation that the above phenomena are due to the effects of these alkaloids on the rate of absorption of glucose from the intestine.

Further points of interest are the following :

There is no unanimity as to the exact region from which maximum absorption of glucose takes place. According to some investigators it is the duodenal region, others think it to be the jejunum, while some opine it to be the ileum (18-22). Verzar and Wirz (23) found that in the rat, the rate of the absorption of glucose is 30% faster in the upper part of the intestine than in the lower. Xylose which is not selectively absorbed, is absorbed at the same rate along the entire intestine. The present investigation however shows that in the quinea-pig, though the rate of absorption of xylose is uniform all over the intestine, glucose is absorbed 26% faster in the ileac end than at the duodenal (Table I).

A difference in the relative absorption rate of glucose and xylose from intestine may be noted in different species of animals. Cori (2) found that in rat intestine, the ratio of absorption rate of glucose to that of xylose is 100:15. Miller and Lewis (24) noted the absorption of 166 mg. of glucose and 29 to 46 mg. of xylose per 100 g. per hour from rats' intestine. Verzar (5) found from the intestinal loops of rats that an isotonic glucose solution is absorbed 3 to 4 times faster than an isotonic xylose solution. Davidson and Garry (25) however could not had this difference in the absorption rate of glucose and xylose in ileum of cat. In this investigation we have noted that in guinea-pigs, an isotonic glucose solution on an average is absorbed 1.4 times faster than isotonic xylose solution. In the ileum, however, the absorption rate of isotonic glucose solution was found to be 1.7 times faster than an isotonic xylose solution.

SUMMARY

The effects of quinine, atropine, and strychnine on the rates of absorption of glucose, fructose, xylose and arabinose have been studied.

(1) Quinine increases the rate of glucose absorption by 46% but has no influence on fructose, xylose and arabinose.

(2) Atropine reduces the rate of absorption of all the sugars.

Diet: Yellow Corn   ...   . 57 g.
       Linseed meal   12
       Milk powder (Glaxo)   25
       Casein 3.7
       Salt mixture (Osborn and Mendell)   ... 1.6

Animals belonging to group II were used to study· the process of absorption from the intestine by the direct method. These were kept without food for 30 hours before starting the actual experiments, but were allowed to drink water *ad libitum* and at the end of the fasting period, 1.3 mg. of urethane per g. of body weight was injected intramuscularly. After a couple of hours, when the animal was completely anæsthetised, the abdominal cavity was opened and a loop 40 cm. in length was tied up—the first knot was given about 3 cm. below the point at which the bile and pancreatic ducts enter, thus completely occluding these two secretions from the region of absorption. After an hour, during which period, the animal was kept at 37°C to recover from the shock of the operation, the test solutions, containing the following substances were introduced into the loops by means of a fine hypodermic needle.

Solution A.	Solution B.	Solution C.	Solution D.
1 cc. olive oil. 5 cc. of a solution containing 25 mg. of bile salt and 0.2 g. Na-glycerophosphate per cc. 2.5 cc. of a lipase solution containing 50 mg. lipase per cc. 0.5 cc. water.	0.5 cc. water in solution A was replaced by 0.5 cc. of quinine-HCl containing 10 mg. of quinine-HCl per cc.	1 cc. olive oil in solution A was replaced by 1 cc. fatty acid and lipase solution was replaced by an equal volume of water.	Olive oil and water in solution A was replaced by 1 cc. fatty acid and 0.5 cc. of quinine-HCl solution. Lipase solution was replaced by an equal volume of water.

1.5 cc. of the above solutions was introduced into the intestinal loops and the animals were again kept at 37°C ; after 3 hours these were sacrificed and the intestinal contents were washed out by a jet of alcohol-ether mixture. The washings were collected in a beaker and this was filtered in an Erlenmayer flask and the beaker was washed twice with 5 cc. of the above mixture and filtered as before. The combined mixture was reduced to a volume of 5 cc. and neutralised with alcoholic-potash (0.5N). The neutralised solution was then reduced to a volume of 2 cc. and saponified with 5 cc. alcoholic-potash (0.5N) for 1½ hours and then titrated with 0.1N H₂SO₄, using phenolphthalein ·as the indicator. The intestinal mucosa of the above animals were scraped and dried at 110°C and definite weights of the dried mucosa was refluxed in a Soxhlet apparatus. The ethereal extract was then neutralised and saponified as before. ' The fæces of the 3 subgroups of Group I were also dried at 110°C and then refluxed with ether. The ethereal extract was treated as before.

Animals belonging to Group III were kept without food for 30 hours. These were then killed with a blow on the head. The intestinal content was washed out by ether-alcohol mixture and the intestinal mucosa was scraped out, dried and treated as before to determine the amount of fat present in the intestinal content and the intestinal mucosa of fasting rats.

The findings are summarised in the following tables.

TABLE I

The values of fat are given in terms of N/10 fatty acid.

Absorption period 3 hours.

Each experiment was conducted in group of 3 rats.

The intestinal contents of 3 fasting rats contain 0.42 cc. of N/10 free fatty acid and no neutral fat.

The amount of fat absorbed is equal to, (amount introduced in the intestine + amount present in fasting rats) − (amount recovered from the intestine).

Experiment No.	Solution used.	Amount introduced.	Amount recovered from the intestine after the absorption period.		Amount absorbed.	Percentage of absorption.
			Fatty acid as neutral fat.	Free fatty acid.		
1	A	19.78	5.8	2.58	11.82	60%
2	B	17.13	11.88	0.11	5.53	37
3	C	4.18	—	2.57	2.03	48.5
4	D	4.33	—	3.02	1.73	40

TABLE II

Neutral fat and free fatty acid content of the dry intestinal mucosa in post-absorption and fasting conditions. The experiments were conducted in groups of 3 rats. The values of fat are given in terms of N/10 fatty acid.

Nature of Solution used.	Free fatty acid per g. of intestine.	Neutral fat per g. of intestine.	Free fatty acid. Neutral fat.
A	3.66	13.98	0.262
B	2.61	8.68	0.301
C	8.06	10.68	0.755
D	6.00	7.9	0.76
Fasting	2.09	2.3	0.9113

TABLE III

Fat content of 24 hour fæces. The experiments were conducted in groups of 2 rats and the results are given as such. As usual, the values of fat are given in terms of N/10 fatty acid. Fat content of diet was 48.6 cc. of N/10 fatty acid.

Group Number.	Fat excreted in fæces.	Fat absorbed.	Percentage of absorption.
A	6.7	41.9	86
B, (10 mg. quinine-HCl intravenously)	6.6	42	86
B, (15 mg. quinine-HCl intravenously)	,,	,,	,,
B, (20 mg. quinine-HCl intravenously)	,,	,,	,,
B, (10 mg. quinine, alkaloidal, per os)	7.95	40.6	83
C, (10 mg. quinine, alkaloidal, per os)	8.00	,,	,,
C, (15 mg. quinine, alkaloidal, per os)	10.75	37.9	78
B and C, on stoppage of quinine administration	6.6	42	86

DISCUSSION

Quinine is known to be a specific poison of lipase, even such small quantities as 2 mg. stops lipase activity (1). It is however quite interesting to find that large amounts of fat are absorbed even when the diet is mixed with this alkaloid (table III). The animals were taking in fat equivalent to 48.6 cc. N/10 fatty acid. (It should be borne in mind that the ether soluble material present in the intestine or fæces contain beside true fats and fatty acids, other organic compounds like sterols, etc., and therefore expressions in terms of weight can never give a true indication of the extent of absorption or excretion of true fats. We have therefore expressed the contents in terms of N/10 fatty acid). The normal fat excretion was 6.7 cc. and this rose to 8 cc. when 10 mg. of quinine alkaloid was mixed with the diet. This rise is immediate, that is, the effect is exhibited in the sample of fæces collected on the following day. The diet was given at 11 A.M. and 6 P.M. everyday and fæces was collected at 11 A.M. When the diet was mixed with 15 mg. of the alkaloid the excretion rose to 10.75 cc. The excretion became normal when quinine was withdrawn from the diet. It was not possible to increase the amount of quinine in the diet beyond 15 mg., nor' was it possible to continue the experiments for any length of time. The animals refused to take their meal from the fourth day of the commencement of the administration of quinine per os. Quinine-HCl was also given to a group of rats intravenously. At first 10 mg. of it was injected for 5 days and this was without

any effect ; the dose was increased to 15 and 20 mg. Even these toxic doses were without any effect on the excretion of fat in the fæces. But when these animals were given 10 mg. of alkaloidal quinine per os, the excretion rose immediately from 6.6 to 7.95 cc.

It was decided at this stage to study the absorption of neutral fat and free fatty acid directly from the intestine in presence of quinine. The results of this series are collected in the table I. It will be seen that normally 60% of fat is absorbed in 3 hours from the intestine ; but when quinine-HCl was also injected into the intestinal loops, only 37% was absorbed. The analysis of the intestinal contents revealed that the percentage of free fatty acid is 30.7% of the total fatty acid in the normal and 1.1% when quinine was also injected in the intestine. It appeared therefore that quinine was inhibiting the rate of hydrolysis of fat by lipase and hence the decreased rate of absorption. This assumption is however quite logical in view of the fact that lipase activity is stopped in vitro experiments (1). Another set of experiments were carried out, namely the absorption of fatty acids from the intestine. (The fatty acid sample was prepared by saponifying a certain quantity of olive oil. The fatty acid was liberated from the saponified mass by the addition of HCl and the liberated fatty acid was extracted by petroleum ether, which was subsequently evaporated out). It was found by Verzar and Laszt (2) that fairly large amount of fatty acid is absorbed in presence of Na-glycerophosphate. This compound was therefore included in all the experiments conducted. It was found that 48.5% and 40% of fatty acid was absorbed from the normal and quinine treated intestines respectively. The decreased rate of absorption seems to be due to the toxicity produced by higher concentration of free fatty acid itself (3) ; the 8.5% decrease in presence of quinine may be explained thus: quinine removes a certain percentage of the bile acids, in fact it becomes a competitor to fatty acid, for its own absorption (4). Whereas in the former case, all the bile acids were being utilised for the absorption of fatty acids only, the same amount of bile acid was used for the absorption of both fatty acid and quinine in the latter and hence the above decrease of 8.5% in the rate of absorption. These experiments also explain tentatively the mechanism through which the enormous decrease in the absorption of neutral fat in presence of quinine occurs ; the explanation is that lipase activity was inhibited due to the presence of quinine causing thereby decreased hydrolysis of fat which in its turn was responsible for the fall in the rate of absorption. The behaviour of quinine towards the absorption of fat is entirely different from that of mono-iodoacetic acid or phlorrhizin. Mono-iodoacetic acid not only stops the breakdown of fat in the intestine, it also inhibits the resynthesis of fat in the mucosa by poisoning the phosphorylation mechanism (5). Phlorrhizin has no effect on the hydrolysis of fat but it inhibits absorption by poisoning the phosphorylation process (6). It will be evident from the analysis of the intestinal mucosa that quinine does not stop the resynthesis of fat and therefore has no appreciable action on the absorption of fatty acids. The ratio of Fatty acid/Neutral fat in the intestinal mucosa does not change ; whereas the ratio was 0.755 in case of fatty acids only, it was 0.76 in those cases where quinine

was also administered.   Moreover, injection of mono-iodoacetic acid in the blood stream also causes a complete inhibition of fat absorption (5) but the picture is quite different in case of quinine.   Even when toxic doses of 100 mg. per kg. of body-weight are injected intravenously, the absorption of fat proceeds quite normally.

The assumption therefore that the inhibition of neutral fat absorption in presence of quinine is due entirely to the effect of this alkaloid on hydrolysis and not on the phosphorylation mechanism, or to be more precise, the resynthesis of fat in the intestinal mucosa, is very conclusively proved from what has been stated.

The question naturally arises, why such large amount of fat present in the diet is absorbed even when quinine was mixed with the diet itself?   It will be seen from what has been said before that both the pancreatic and bile secretions from their respective glands were stopped from coming into the region of absorption and a limited amount of lipase was available for the hydrolysis of fat in those cases where the process of absorption was studied direct from the intestine.   But in those animals where quinine was administered per os, lipase and bile were being continuously secreted and quinine was also gradually removed from the intestine by the process of absorption due to the hydrotopic action of bile salt— thereby allowing the hydrolysis and absorption of fat to proceed.   Only in those cases where enormous quantity of quinine was given, the normal rate of absorption was hampered to some extent.

SUMMARY

The effect of quinine on the absorption of fat has been studied.

It has been found that a decrease of 23% and 8.5% in the rate of absorption from the intestine of neutral fat and fatty acids respectively, take place in presence of quinine, while analysis of faeces shows that the excretion of fat does not change even when 100 mg. of quinine-HCl per kg. of body-weight is injected intravenously but the same rises when 50 mg. of alkaloidal quinine is given per os.

Estimations of the different components of fat of the intestinal contents and the intestinal mucosa post-absorptively reveal that quinine inhibits the rate of absorption by decreasing the rate of hydrolysis and unlike, mono-iodoacetic acid and phlorrhizin, it has no effect on the resynthesis of fat in the mucosa.

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STUDIES ON THE EFFECT OF CERTAIN EXPERIMENTALLY INDUCED PHYSIOLOGICAL DISTURBANCES ON THE BIOSYNTHESIS OF ASCORBIC ACID BY THE RAT.

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Recent observations by Longenecker, Fricke and King (1) on the increased excretion of ascorbic acid by the rat, when fed with certain nerve depressants, have opened a new line of approach to the study of the mechanism of biosynthesis of ascorbic acid. They regarded the excess of ascorbic acid excreted by the animal under the influence of certain narcotics to be due to increased synthesis of ascorbic acid in the body and its quick disposal by the kidney glomerules (2). This view seemed to be in agreement with the non-reabsorptive capacity of the kidney glomerules for ascorbic acid. The mechanism of this synthesis is, however, not yet understood. These nerve depressants cannot conceivably serve as direct precursors in the synthesis of ascorbic acid, but may probably affect the transformation of some intermediary metabolites. In order to throw light, if possible, on the mechanism of this transformation, attempts have been made to study the mechanism of ascorbic acid formation under certain physiological conditions which may perhaps be, to some extent, analogous to the conditions produced by nerve depressants. Experiments have been carried out to investigate the urinary excretion of ascorbic acid (both free and combined) and also the content of tissue ascorbic acid under the following conditions *viz.* (1) during vitamin B_1-deficiency, (2) during anoxia due to lack of oxygen supply and during anæsthesia, (3) during hypoglycemia produced by insulin injection and (4) during poisoning of the respiratory enzymes by cyanide and malonate.

I. EFFECT OF VITAMIN B_1-DEFICIENCY

During vitamin B_1-deficiency in the rat the oxygen consumption of the brain is diminished along with it. The metabolism of normal carbohydrate breakdown is also disturbed. Peters and others (3) have shown increased accumulation of

pyruvic acid in the brain of avitaminous rats, Harper and Deuel (4) have observed unoxidised pyruvic acid excretion in the urine of rats during vitamin B_1 deficiency. Narcotics are considered to behave similarly in affecting the normal oxygen uptake and the normal carbohydrate breakdown. In order to investigate the mechanism of increased ascorbic acid excretion by narcotised rats, experiments have been carried out to observe the effect of vitamin B_1-deficiency in rats on their urinary excretion of ascorbic acid (both free and combined).

Rats weighing between 40 and 60 g. were divided into two groups. One group of rats was given a vitamin B_1-deficient diet (5) which consisted of dextrinised starch 79 parts, Casein 15 parts, agar-agar 2 parts, Steenbock salt mixture, No. 40, 4 parts and yeast autoclaved at 15 lb. pressure for 6 hours (5) 25 parts. The control group of rats was supplied with dextrinised starch 73 parts, casein 15 parts, salt mixture 4 parts and dried yeast 8 parts. Each animal of both the control and vitamin B_1-deficient groups was given a supplement of 4-5 drops of the codliver oil twice a week. Each animal was kept in a separate metabolism cage and the urinary excretion of both free and combined ascorbic acid was determined at intervals of 4 days by the method previously described (6). The experiment lasted for 5 weeks when the vitamin B_1-deficient rats were almost in the dying condition. The results are given in Table I.

TABLE I

No. of Expt.	Condition.	Rat No.	Average daily excretion of ascorbic acid per animal per day expressed in mg.		
			Total.	Free.	Combined.
I.	Control.	1.	0.082	0.053	0.029
		2.	0.0657	0.0446	0.0211
		3.	0.0914	0.0560	0.0351
		4.	0.0777	0.0487	0.0290
		5.	0.0990	0.0605	0.0385
		Mean.	0.0872	0.0526	0.0305
II.	Vitamin B_1-deficient	1.	0.0762	0.0561	0.0202
		2.	0.0682	0.0500	0.0182
		3.	0.0812	0.0600	0.0212
		4.	0.0637	0.0192	0.0145
		5.	0.0610	0.0115	0.0195
		Mean.	0.0700	0.0516	0.0187

TABLE II

No.	Mg. of ascorbic acid per g. of fresh tissue.		
	Brain.	Liver.	Kidney.
I. Control (average of 5 rats)	0.333	0.180	0.170
II. Vitamin B₁-deficient (average of 5 rats)	0.318	0.161	0.172

No difference in the excretion of free ascorbic acid was observed, only the vitamin B₁-deficient rats showed decreased excretion of combined ascorbic acid. It is clear therefore, that the mechanism of increased excretion of ascorbic acid in the urine of narcotised rats is quite different from that produced by simple vitamin B₁ deficiency.

The amount of ascorbic acid present in the brain, liver and kidney tissues of rats (both normal and deficient) has been investigated. The results in Table II showed that brain and liver tissues of the deficient animal suffered loss in ascorbic acid content while the kidney tissue showed no difference.

2. EFFECT OF ANOXIA OR LACK OF OXYGEN SUPPLY

McClure et al (7) have demonstrated the existence of anoxia in the central nervous system during the action of narcotics. Anoxia produced by simple decrease in the oxygen supply can affect the normal respiration and to a certain extent the normal metabolism. Experiments have been carried out to investigate the effect of anoxia on the excretion of ascorbic acid (both free and combined). Anoxia in the rat was produced by allowing rats to breathe inside a closed vacuum desiccator. Rats weighing between 125 g. and 200 g. were placed separately inside a desiccator which contained a plate full of caustic soda at the base to absorb CO_2 produced from the expiration of the experimental animal. A porcelain basin fitting the porcelain base of the desiccator was placed inside it. The basin contained 10 drops of dilute H_2SO_4 (1:4) in which the urine of the experimental animal was collected. After a period of 6 hours, when the animal was in a moribund condition, it was removed from the desiccator and killed by a blow.

The ascorbic acid content of the urine was estimated (Table III). The amount of ascorbic acid present in the tissues like blood (drawn from the heart), brain. liver and kidney was determined and compared with that obtained from the rats killed by ether, chloroform and coal gas. Results are given in Table IV.

It will be observed from Table IV that anoxia due to simple deprivation of oxygen and ether anæsthesia cause increase in the concentration of ascorbic acid in the liver and kidney tissues to some extent as chloretone does (8) Chloroform produced practically no effect on the concentration of ascorbic acid. Coal gas diminished the concentration of ascorbic acid in the· brain tissue considerably.

TABLE III

Urinary excretion of free and combined ascorbic acid in rats during anoxia and in normal condition.

Condition.	Mg. of ascorbic acid excreted per animal during 6 hours.	
	Free.	Combined.
Anoxia (average of 3 rats)	.096	.039
Normal (average of 5 rats)	.095	.058

TABLE IV

Tissue ascorbic acid.

No.	Condition.	100 cc. blood.	Mg. of ascorbic acid per g. of fresh tissue.		
			Brain.	Liver.	Kidney.
1.	Normal	1.66	0.316	0.185	0.164
2.	Anoxia	1.66	0.300	0.190	0.193
3.	Ether	1.88	0.335	0.216	0.235
4.	Chloroform	1.60	0.301	0.180	0.161
5.	Coal gas	—	0.265	0.197	0.176
6.	Chloretone	1.61	0.338	0.279	0.252

3.   Effect of Hypoglycemia due to Insulin Injection

During the hypoglycemia induced by insulin injection there is decreased consumption of oxygen by the brain and lessened glucose utilisation by this organ. Himwich *et al* (9) have demonstrated that by injecting insulin, various neurological disturbances are produced in the system comparable with those of anoxia. It therefore, seemed desirable to know whether the injection of insulin into normal rats, can produce increased excretion of ascorbic acid in the urine. The results (Table V) obtained by the injection of one and two units of insulin showed, however, no appreciable variation regarding the urinary excretion of free and combined ascorbic acid.

TABLE V.

Mg. of ascorbic acid excreted per animal for a period of 4 days before and after insulin injection.

Rat No.	Insulin injected	Free ascorbic acid.		Combined ascorbic acid.	
		Before.	After.	Before.	After.
1.	1 unit	0.512	0.564	0.358	0.336
2.		0.404	0.401	0.379	0.316
3		0.272	0.268	0.368	0.352
4.	2 units	0.450	0.450	0.330	0.310
5.		0.666	0.618	0.367	0.367
6.		0.502	0.131	0.351	0.319

4.   Effect of Respiratory Poisons

Cyanide and malonate are regarded to be respiratory poisons, cyanide inhibiting the cytochrome system and the other the succinodehydrogenase system.

(a)   Action of cyanide

Administration of cyanide produces anoxia which is regarded to be due to the inhibition of the respiration by blocking the respiratory enzyme. It was therefore interesting to study the effect of sub-lethal doses of cyanide on the urinary excretion of ascorbic acid.   The results which are tabulated below (Table VI) showed decrease in both free and combined ascorbic acid excretion as a result of cyanide administration.

TABLE VI

Mg. of ascorbic acid excreted per animal for a period of 4 days
before and after 0.5 mg. of cyanide injection.

Rat No.	Free ascorbic acid.		Combined ascorbic acid.	
	Before.	After.	Before.	After.
1.	1.120	0.830	0.110	0.070
2.	0.733	0.654	0.562	0.326
3.	0.564	0.536	0.169	0.134
4.	0.587	0.542	0.443	0.430

(b) Action of malonate

Malonate is considered to be a respiratory poison as it inhibits the oxidation of succinate by succinodehydrogenase. According to Greville (10) malonate brings about a decrease in the oxygen consumption of the rat's brain even in the presence of glucose. Recent experiments by Krebs (11) and by Smith and Orten (12) have indicated that the injection of malonate can give rise to increased synthesis of citric acid. Whether ascorbic acid can be produced along with citric acid appeared to be a question worthy of study. The effect of intraperitoneal injection of 20 mg. of neutratised malonic acid on the urinary excretion of both free and combined ascorbic acid was therefore studied. The results in Table VII indicate no variation in the excretion of ascorbic acid before and after malonate injection. Therefore, it may be concluded that the path of citric acid formation is quite different from that of ascorbic acid synthesis.

TABLE VII

Mg. of ascorbic acid excreted per animal for a period of 4 days
before and after injection of 20 mg. malonic acid.

Rat No.	Free ascorbic acid.		Combined ascorbic acid.	
	Before.	After.	Before.	After.
1.	0.281	0.233	0.379	0.217
2.	0.312	0.310	0.478	0.440
3.	0.561	0.450	0.246	0.228
4.	0.418	0.377	0.218	0.219

DISCUSSION AND SUMMARY

In order to throw light on the mechanism of formation of ascorbic acid in narcotised rats, the effect of certain physiological disturbances analogous to those produced by the action of narcotics has been studied with reference to the excretion of ascorbic acid and the content of tissue ascorbic acid.

Physiological changes due to vitamin B_1-deficiency in the rat have not produced any appreciable change in the ascorbic acid excretion. The ascorbic acid content of the brain and liver tissues was decreased in vitamin B_1-deficiency, while the ascorbic acid content of kidney tissue remained unaltered.

Anoxia produced by the lack of oxygen supply did not appreciably increase the excretion of ascorbic acid in the urine of rats. The concentration of ascorbic acid in the kidney tissue was however, increased showing some resemblance with the effect of narcotics (14). Ether anoxia showed increase in the concentration of ascorbic acid in all the tissues especially the kidney. The ascorbic acid content of the liver and kidney was found to be increased in etherized rats by Bowman and Muntwyler (13) who also observed a small increase in the urinary excretion (14). Unfortunately the urinary excretion of ascorbic acid under etherised condition could not be studied owing to experimental difficulties. Chloroform and coal gas seemed on the other hand to decrease the tissue content of ascorbic acid. Ascorbic acid concentration in the blood under these different conditions induced by anoxia, ether, chloroform, coal gas and chloretone showed no significant variation except perhaps in the etherised condition in which the ascorbic acid level of blood seemed to be slightly increased. The increase in the concentration of ascorbic acid in the kidney tissue suggested two possibilities. One, the kidney tissue may synthesise ascorbic acid from carbohydrate metabolites, and the other, ascorbic acid may be drawn from the blood stream and locally concentrated in the kidney tissue. The re-absorptive mechanism for ascorbic acid appears to be limited by a maximum rate so that when ascorbic acid is presented to the tubules by the glomerular filtrate at a rate exceeding the maximum, the excess is excreted in the urine (15). Although ascorbic acid is related to sugar in structure it is not reabsorbed by the same mechanism as sugar and ascorbic acid appears far more readily in the urine than ingested sugar.

Insulin injection made hardly any difference in the urinary excretion of ascorbic acid.

The effect of cyanide injection showed decrease in both free and combined ascorbic acid excretion in the urine of rats.

Malonate injection produced slight decrease in the excretion of free ascorbic acid by the rats. The mechanism of ascorbic acid synthesis is therefore not analogous to that of citric acid.

Combined ascorbic acid was found to be excreted as usual under these psysiological disturbances.

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CYANIDE DETOXICATION IN THE RABBIT AND THE DOG AS MEASURED BY URINARY THIOCYANATE EXCRETION

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This paper embodies one aspect of the findings obtained in a comprehensive study of the problem of cyanide poisoning and the mechanism of antidotal action in this condition. The main observations in this study have already been published by Mukerji (1), Smith and Mukerji (2), Smith, Mukerji and Scabury (3), and Mukerji and Ghosh (4). The indications afforded by these data however are of sufficient interest to merit presentation as an independent piece of investigation.

It has long been known that an important physiological mechanism of cyanide detoxication is by thiocyanate formation. More recent and detailed investigations on quantitative thiocyanate excretion after cyanide administration by Hunt (5), Dezani (6), Bodansky and Levy (7), Bodansky (8), Smith and Malcolm (9), Lang (10), and Stuber and Lang (11) have given additional support to the same view. It is now generally accepted that thiocyanate (SCN) formation regularly takes place in the animal organism from the intermediate product (HCN) ingested through the agency of foods and spices rich in cyanogenetic bodies. This conversion of a highly toxic principle to a comparatively non-toxic substance appears to be a defence measure of the body similar to the detoxication in the liver of indol into indican, a normal urinary constituent.

Working on this hypothesis, sublethal amounts of sodium cyanide (NaCN) were injected into a series of rabbits and dogs and the urinary elimination of thiocyanate was measured from day to day up to eight days in the majority of experiments. The data, obtained by more accurate methods than those available to many early workers, not only confirm the usual conception of the importance of thiocyanate formation in cyanide detoxication but point unequivocally

to certain differences in the reactions of rabbits and dogs to cyanides introduced into their systems, which promise to throw newer light into certain obscure metabolic processes.

EXPERIMENTAL

PROCEDURE:

Experiments were performed with a total of 65 rabbits and 15 dogs. The tables appended, however, contain detailed data of the representative experiments only.

Healthy rabbits (mostly females and litter mates) weighing between 2 and 3.5 kilos were chosen from an in-bred colony. They were kept, for a week preceding and throughout the experimental period, on a constant daily diet of 100 g. lettuce, 100 g. carrots and 100 g. of equal parts of oats and/or alfalfa hay or 'Purina' rabbit chow. Cabbage was avoided as it is known to contain a fairly large amount of SCN. By preliminary trial, the diet allowed was found to be almost completely utilized and was sufficient to maintain their body weights. Water was allowed in measured quantities once daily.

Dogs used weighed between 6 to 8 kilos and were allowed a constant daily diet of 'Purina' dog chow, although the amount varied with individual animals. Female dogs were used exclusively to avoid any urine wastage.

Cages designed for metabolism experiments and suitable for the collection of urine were used for both rabbits and dogs. Precautions were taken to avoid any spilling of water on the floor of the cages and consequent interferences with the total urine volume. A normal control period of 5 to 7 days was allowed before experiments were started. Samples of urine, 24 or 48 hours, were collected during this period for analysis. After experimental treatment, the first collection was sometimes made between 40 and 48 hours. The collections represent spontaneously voided urine without bladder compression.

In a few *acute experiments*, artificial diuresis was induced in anæsthetised rabbits (morphine + urethane) by continuous perfusion (rate of 1 cc. per kilo per min.) of sodium sulphate (1%) in normal saline through the external jugular vein. The urinary bladder was canulated and half hour urine collections were made before and after the sodium cyanide and thiocyanate injections. More or less uniform collections could be obtained from such a preparation for a period of 4 to 5 hours. Intraperitoneal œdema later prevents satisfactory results.

Standardized solutions of NaCN and NaSCN (C.P.) were used in all experiments, the usual dosage for the former being 1 mg. in N/10 solution over a period of approximately 5 minutes. The injections were made in the ear vein of rabbits and in the hind leg vein of dogs unless otherwise stated. In comparative experiments, equivalent amounts of NaSCN were always employed.

METHODS:

A number of methods are described in the literature for the determination of SCN in biological fluids. Many of these, however, are not reliable quantitative methods. The Rupp-Schied-Thiel method (12) depending on iodimetric titration was considered the best quantitative method and was used by most of the early

investigators. Sullivan and Hess (13) and Baumann *et al* (14) have shown that this method is not specific for SCN and determines ergothioneine-like material, proteoses, sulfides, mercaptans, etc. The values obtained therefore are usually far in excess (20-2,000% too high) of the amounts actually present. A modification of the method has been described by Sullivan and Hess (*loc. cit.*) and two new procedures of greater specificity have also been recently described. (Baumann *et al, loc. cit.*; Hartner (15),). Both these methods have been employed by us in the determination of thiocyanate in the urine. The complete procedure of Baumann *et al* was generally employed but a smaller series of determinations were done according to Hartner method side by side with serum thiocyanate determinations required for another aspect of our comprehensive investigation. No direct comparison has been made between these methods but the results of thiocyanate recoveries from urine were quite satisfactory and the errors seldom exceeded 5 per cent.

Duplicate determinations were done in each case and control determinations were run side by side in every experiment.

RESULTS:

1. *Rabbit experiments*

(a) Normal level of Thiocyanate excretion:—The normal SCN elimination under standardized conditions of diet and laboratory environment was first established. A consideration of the data on 38 experiments on 24 or 48 hour urine collections showed wide variations not only between individual rabbits but also between day-to-day SCN excretions of the same rabbit. In extreme cases a difference in the daily SCN elimination of 100 to 400 per cent. might be found. The variation in the SCN values from day-to-day, in the same animal, however, was much less marked. It appears likely that SCN metabolism is dependent on factors other than diet alone.

The average daily SCN excretion in 35 rabbits was found to be 0.63, S.D. ± 0.26 mg. This value is higher than that of 0.3 to 0.4 mg. reported by Baumann *et al* (1934), which may be due to the larger size of our rabbits (approximately 3 kg.) and to the more ample diet.

(b) Thiocyanate excretion after NaSCN administration:—The relative proportion of SCN excretion following NaSCN injections was next established with a view to find out if thiocyanate *per se* has any influence in altering the normal thiocyanate metabolism. Table I represents a typical series of experiments. It will be seen that within 92-96 hours following an injection of NaSCN, about 90% may be recovered on an average. (21 experiments—91.0%, S.D. ± 10.72). This of course included the endogenous thiocyanate excretion also for the period under observation. Even if we eliminate this amount, the evidence from the data is strong enough to indicate that there is an almost complete clearance of NaSCN through the kidneys. It has been sometimes suggested that a part of SCN is oxidized and transformed in the body. If this occurs at all, it must be an insignificant amount.

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TABLE I

Thiocyanate Excretion After Intravenous NaSCN (N/50 Sol. 1cc./kg.)

Thiocyanate Recovery in Urine.

Rabbit No.	Weight.	Quantity injected expressed as SCN.	1st Collection (10 hrs.)			2nd Collection (68 hrs.)			3rd Collection (96 hrs.)			4th Collection (150 hrs.)		
			Urine Vol.	SCN.	Total.	Urine Vol.	SCN.	Total.	Urine Vol.	SCN.	Total.	Urine Vol.	SCN.	Total.
1	3.6 kg.	20.8 mg.	168 cc.	16.4 mg.	79%	271 cc.	21.7 mg.	104%	444 cc.	22.5 mg.	119%	714 cc.	24.8 mg.	119%
2	2.7	15.6	88	7.7	19	163	17.0	109	310	18.7	120	390	19.4	124
3	3.65	21.2	50	8.1	38	67	16.1	76	155	20.8	98	225	21.7	102
4	3.75	21.7	130	5.1	24	213	13.2	61	315	15.3	71	315	17.6	81
5	3.35	19.4	125	12.2	63	160	16.1	83	292	17.0	88	507	18.1	95
6	2.6	15.1	150	11.1	75	220	17.6	116	150	18.2	120	710	19.1	126
Average (% excretion)					55			91.5			100.1			108.5

N.B.—The thiocyanate excretion represented in the table is *relative* and includes also the endogenous thiocyanate excretion of the animals for the corresponding period. This is the reason why the percentage excretion has gone up above 100 in 96 hrs. and 150 hrs. collection.

(c) Thiocyanate recovery after cyanide administration:—Tables II, III and IV give representative group of experiments designed to show the conversion of injected (intravenous injection, Table II ; subcutaneous injection, Table III) cyanide into thiocyanate. For this purpose, stoichiometrically equivalent quantities of NaCN and NaSCN were injected in the same group of rabbits at different times and by different routes of administration. A few experiments were also performed under identical conditions in rabbits with artificially induced diuresis. A comparison of the (i) and (ii) columns in the tables II & III shows parallelism between CN and SCN excretion, suggesting an almost complete conversion of CN into SCN. The average SCN excretions in the urine during the first 40 hours and 66 hours are slightly higher in (i) in both Tables II and III but the differences are not statistically significant. It is difficult to say how much CN is actually converted, but the effect is approximately the same for equimolecular amounts of the two compounds, e.g. NaCN and NaSCN. This is also seen from Table IV where artificial diuresis was induced and urinary SCN recovery was recorded for a comparatively short period of $4\frac{1}{2}$ hours.

TABLE II

SCN recovery after CN administration

(i) NaSCN (Equivalent of 2 mg./kg. NaCN) intravenously.

Rabbit No.	Body weight.	Amount injected expressed as SCN.	SCN Recovery in Urine.					
			Collection I (40 hrs.)		Collection II (66 hrs.)		Collection III (96 hrs.)	
			SCN.	Total.	SCN.	Total.	SCN.	Total.
35	3.3 kg.	7.8 mg.	5.9 mg.	76%	7.9 mg.	90%	7.5 mg.	95%
36	2.9	6.9	5.3	77	6.2	90	6.9	100
37	4.5	10.6	6.4	60	7.5	71	8.1	79
38	3.05	7.2	3.3	46	4.4	61	5.0	70
39	3.1	7.3	5.1	70	5.7	78	6.0	82

Av. (% excr.)+S.D.=66±11.6   78±11.8   82

(ii) NaCN (2 mg./kg. intravenously)

35	3.1	7.3	3.8	52	4.7	64	5.4	74
37	4.25	10.0	7.5	75	9.1	91	10.7	107
38	3.1	7.3	3.0	41	3.4	46	4.3	59
39	2.85	6.7	4.4	66	5.1	75	6.0	88
40	3.2	7.6	4.9	65	6.1	80	7.3	96

Av. (% excr.)+S.D.=60±11.91   71.2±15.3   82

TABLE III

(i) NaCN (subcutaneously)

| Rabbit No. | Weight. | Amount as SCN. | SCN recovery in Urine. | | | | | |
			Collection I (40-18 hrs.) SCN.		Collection II (90-96 hrs.) SCN.		Collection III (144 hrs.) SCN.	
47	2.75 kg.	16.2 mg.	10.3 mg.	61%	11.22 mg.	69%	11.88 mg.	73%
48	2.95	17.4	18.6	61	11.44	69	12.42	74
49	2.85	16.8	8.4	51	8.98	54	9.82	59
50	1.95	11.5	7.76	67	8.33	73	9.09	79
51	2.20	13.0	8.66	67	9.67	75	10.55	82
Mean+S.D.			62.0 ± 5.46		68	-	73.4	

(ii) NaSCN (subcutaneously)

47	2.80	8.10	4.68	58	5.27	65	5.76	72
48	3.15	9.15	6.05	66	6.73	74	7.21	79
49	3.05	8.85	4.76	51	5.22	59	5.68	65
50	2.10	6.10	3.49	57	3.87	61	4.38	72
51	2.15	7.10	4.19	59	4.63	66	5.01	71
Mean+S.D.			58.8 ± 3.97		65.6		71.8	

TABLE IV

SCN elimination during artificially induced diuresis of rabbits.

Total SCN recovery in $4\frac{1}{2}$ hours.

Treatment.	Amount injected as SCN.	Normal SCN (30 min. recovery before inj.)	SCN recovery during and up to $4\frac{1}{2}$ hours after injection.	
NaSCN (Equivalent of 2 mg./kg. NaCN)	5.6 mg.	0.093 mg.	0.46 mg.	8.2%
	6.3	0.093	0.56	8.9
NaCN	7.26	0.17	0.54	7.4
	7.35	0.17	0.65	8.9

TABLE V

Urinary Thiocyanate excretion in rabbits before and after 1 mg. per kg. of Sodium Cyanide.

Rabbit No.	Before NaCN 18 hr. Period.		SCN Equiv. of NaCN injected.	After Cyanide.							
				1st 24 hr. Period.		1st 18 hr. Period.		*Recovery.	2nd 18 hr. Period.	
	Vol.	SCN.		Vol.	SCN.	Vol.	SCN.		Vol.	SCN.
60	200 cc.	1.13 mg.	1.25 mg.			200 cc.	3.10 mg.	39 %	220 cc.	1.31 mg.
61	165	1.08	1.15			185	3.63	61	290	0.66
62	205	0.58	3.85	137 cc.	3.35 mg.	300	4.18	93	309	1.02
63	316	1.01	3.55	169	3.26	329	3.71	75	310	1.21
64	325	1.20	1.02	124	3.99	218	4.18	84		
Ave.	291	1.06				232.4	3.82	70.4		

*Percentage recovery is calculated from the thiocyanate excretion in the 48-hour period in excess of the normal excretion.

TABLE VI

Urinary Thiocyanate Excretion in Dogs Before and After 1 mg. per kg. of Sodium Cyanide.

Dog.	Before NaCN 24 hr.		SCN Equiv. of NaCN injected mg.	24-hr. Periods after NaCN.														SCN* Recovery.	
				1		2		3		4		5		6		7			
	Vol. cc.	SCN mg.		Vol. cc.	SCN mg.	Vol. cc.	SCN mg.	Vol. cc.	SCN mg.	Vol. cc.	SCN mg.	Vol. cc.	SCN mg.	Vol. cc.	SCN mg.	Vol. cc.	SCN mg.	mg.	%
1	285	0.72	20.4	215	0.70	320	1.34	385	1.34	555	1.93	245	0.93	130	0.65	430	1.40	3.25	16
2	315	0.85	17.8	212	0.75	310	3.32	280	2.67	342	1.91	300	1.18	250	0.75	300	0.84	5.47	31
3	325	0.45	23.7	225	0.60	410	2.95	215	1.63	220	0.80	230	0.91	300	0.98	225	0.89	5.61	24
4	390	1.09	27.9	510	1.38	270	1.06	285	1.12	195	0.70	230	1.15	315	1.31	255	1.02	0.11	0
5	162	0.56	32.3	340	1.86	150	1.65	370	0.94	170	0.69	345	0.97	172	0.61	225	0.95	3.75	12
Ave.	302	0.73		312	1.06	298	2.06	307	1.54	296	1.21	270	1.03	233	0.86	287	1.02		16.6

*Recovery is calculated from the thiocyanate excretion for seven days in excess of the normal excretion for the same period which was estimated from the 24-hour normal excretion.

In Table V, an attempt has been made in a fresh series of 5 rabbits to estimate the actual amount of SCN excreted in excess of normal rate of elimination after the injections of 1 mg. per kg. of sodium cyanide. Though the rate of SCN recovery is found to vary, an average recovery of more than 70 per cent. is clearly noticeable.

2. *Dog Experiments.*

(*a*) Normal level of thiocyanate excretion:—The thiocyanate excretion for 24 or 48 hour periods in 15 dogs kept under more or less constant dietary and environmental conditions in the laboratory was found to be 0.711 mg., S.D. ±0.38 mg. This figure may be considered as distinctly lower than the corresponding value in rabbits (0.63±0.26 mg.) when considered in terms of the tremendous difference between the two animals in body weights. (Rabbits— approx. 3 kilo. average weight ; dogs approx. 7 kilo average weight).

(*b*) Thiocyanate excretion after cyanide administration:—The rate of SCN recovery after identical treatment with NaCN is seen from Table VI. The administration of cyanide in dogs, unlike the abrupt response seen in rabbits (see Table V), is followed by a moderate rise in SCN excretion, the greatest increase occurring on the 2nd and 3rd days after injection. The excretion continued above normal for at least 1 week which was the limit of our experimental period. Calculated from the daily excretion in excess of the normal value, an average of approximately 17 per cent. of the cyanide was recovered as thiocyanate. Since the excretion was still in excess of the normal, on the 7th day, a prolongation of the experimental period would undoubtedly increase this value. The excess however was so little marked that it was not considered worth while to follow this up systematically.

DISCUSSION

From the data presented above, it appears clear that there are definite 'species differences' as far as thiocyanate formation following cyanide administration is concerned in the rabbit and the dog. In contrast to the prolonged and limited excretion of thiocyanate in dogs, a more abrupt and greater response occurs in rabbits, the average recovery in first 48-hour period after cyanide amounting to above 70 per cent. as against a total of about 20 per cent. during a period of 7 days in dogs. Low recoveries of cyanide as thiocyanate have previously been reported by Lang (17) in one dog (12 per cent.) and by Hug (18) in 3 dogs (48, 28 and 20 per cent.). In these experiments however the dosages were higher than those used by us and determinations were made for 4-day periods only while the excretion was definitely above the normal value which was apparently beyond the range of sensitivity of the methods used.

The question naturally arises as to whether the conversion of CN into SCN may be considered to be the detoxication process of prime importance in both

rabbits and dogs. Evidence presented herein lends strong support to the suggestion that a complete conversion takes place in the rabbit. In view of the somewhat wide fluctuations observed in the normal basal SCN excretion level in experimental animals kept under the most rigid laboratory conditions, an exact quantitative recovery is not possible to obtain, though an effort has been made to arrive at such a figure by subtracting the average normal SCN excretion from the total SCN recovered after CN injection. The parallel SCN excretion with NaCN and NaSCN in the rabbit, under identical conditions, as presented in Tables II, III & IV, is however a cogent indirect proof. This experimental finding is also confirmed by other workers (14, 9).

The extremely low recoveries of SCN in the urine of the dog tend to indicate that SCN formation is at least a process of less importance in this animal. Four possibilities have to be considered in this connection: (a) Elimination by some other channel, (b) Conversion of CN to some other form by oxidation, (c) Slow transformation of CN into SCN, and (d) slow elimination of SCN (already formed) through the kidneys. From reported observations, it appears that elimination through other channels, though perhaps more marked in the dog than in the rabbit, is too small to account for such a pronounced difference. (SCN in dog saliva 0.43 mg. per cent ; in fæces, less than 0.5 mg. daily). Regarding the conversion of CN to some other form as CNO by oxidation, the only evidence available is that of Voegtlin et al (19). This however has not been confirmed later by other workers.

The slow transformation of CN to SCN in such animals as the dog is a possibility which should be given adequate consideration in explaining the differential rate and degree of SCN elimination in rabbits and dogs. In the present experiments, it has been possible by the use of sensitive methods to show that the excretion continues slightly above the normal level for a longer period than that commonly investigated (Lang, (10) ; Hug, (16)). In addition, the prolonged and limited elimination of thiocyanate when it is administered per se to dogs (and also to man) has been a common observation (20) and one which may be related to organic complexes of thiocynate in serum (21). Smith et al (3) recovered the following percentages from one dog after a dose of thiocyanate comparable to the cyanide dosage employed in this investigation: 13 in 48 hours, 29 in 4 days, 57 in 7 days and 74 in 12 days. This points to the fact that SCN formation in the dog is at least a less active process than it is in rabbits.

The present state of our knowledge does not permit any definitely acceptable explanation for this experimental finding. The following evidence however deserves full consideration. Lang (10) demonstrated a much higher 'rhodanase' activity in rabbit liver than in dog liver. This may perhaps be related to the diet, rabbit being a typical herbivorous animal and dog a carnivorous one (4). Tsuru (22) found a more rapid and greater rise in the blood thiocyanate concentration of rabbits than of dogs after HCN administration. Sato's (23) results with nitriles conform with the findings of the two preceding workers.

Smith *et al* (3) determined the values of serum thiocyanate in rabbits and dogs before and after cyanide administration and succeeded in showing a definite rise in serum SCN concentration in both rabbits and dogs. The increase in thiocyanate concentration was not as great or as rapid in the dog as in the rabbit, but it was generally comparable to that in the rabbit.

Such findings on differences in liver activity and serum thiocyanate concentration do not necessarily mean that less cyanide is *ultimately* transformed to thiocyanate in the dog, but if the process is slower in this animal, for which strong evidence is available, it is at least *temporarily* less protected against cyanide intoxication. There is the other possibility of disposal of cyanide by other mechanisms. The remarkable results of James (24) who recovered high percentages of cyanide as such in urine and saliva and on breath after its administration to human subjects suggests that these channels of excretion may play a major part and explain the apparent lack of SCN recovery in urine in the dogs.

SUMMARY

1. By comparatively recent and more reliable methods than those previously used, evidence is presented that cyanide administered either by subcutaneous or intravenous route, is almost completely converted in a comparatively short time into thiocyanate in the rabbit. A very large proportion of the quantity of cyanide injected can be recovered in the urine within the first 24-48 hours after injection or even earlier in experimentally induced diuresis. The excretion rate of SCN following NaCN injection runs closely parallel to that following NaSCN injection in equimolecular amounts.

2. In the dog under identical conditions of treatment, SCN recovery in the urine was very poor indicating a lack of complete conversion of CN into SCN, as in the rabbit. Usually less than 25 per cent. of the injected NaCN can be recovered within a period of 7 days.

3. The question that naturally arises from this finding is whether CN is detoxified through the same mechanism of SCN formation in the dog, as happens in the case of rabbits or whether other methods of detoxication also operate in this animal. The various possibilities in this connection are discussed. Diet by exerting an influence on the enzyme ('rhodanase') activity of the liver may exert an indirect influence. This possibility is being further investigated.

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TOXICOLOGY OF THE FRUITS OF *ZANTHOXYLUM ACANTHOPODIUM* DC

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There are about 35 species of *Zanthoxylum* (*Xanthoxylum*) which have been studied chemically, but only about a half dozen are found in this country. They grow mostly in the temperate Himalayas, in Nepal, Bhutan and in the Khasi hills. *Zanthoxylum acanthopodium* grows wild in the Darjeeling district at an elevation of 5-6000 feet and is locally known as Timbur or Timur. The young leaves are reddish brown in colour with prickles or thorn-like projections on the petioles or stalks. The fruits are of the size of black pepper and contain one or two black seeds. The carpels which resemble coriander, are oval or nearly spherical. Externally they are of bright reddish-brown colour when ripe and covered with prominent tubercles and internally they are furnished with a hard, papery, white membrane which becomes loose, contracts and curls up when the seed falls.

The hill people prepare a kind of *chutney* (sauce) out of this fruit with tamarind, pepper, salt, etc. and take it in very small quantities for treatment of griping and other intestinal disorders. The indigenous systems of medicine prescribe the fruits and seeds in dyspepsia and some forms of diarrhœa, as a drug having astringent, stimulating and digestive properties. "The seeds and the bark are used as an aromatic tonic in fever, dyspepsia and cholera ; the fruit as well as the branches and thorns are used as a remedy for toothache ; also deemed stomachic and carminative and employed to intoxicate fish" (Kirtikar and Basu, 1). It is believed that the fruit possesses poisonous properties as well and produces death if half a dozen are chewed and swallowed in an empty stomach. But so far no authentic case of fatal poisoning by this fruit has been recorded in Darjeeling or elsewhere.

In Darjeeling and in its neighbourhood the green fruits are always available in the bazar in the months of May and June. The peculiar aromatic smell of the fruit and its alleged medicinal properties sometimes tempt the visitors to taste it. It was tasted by one of us and was found to produce an intense tingling sensation on the tongue and on the mucous membranes of the lips and mouth much in the same way as produced by the alkaloids of the aconite root. The leaves, on the other hand, produce no tingling sensation on chewing although they possess the same kind of aromatic smell. It was thought that an alkaloid resembling aconitine or pseudoaconitine (which is responsible for the tingling sensation in the mouth produced by the aconite root) might be present in this fruit as well. With a view to detect the presence of any such active principles this investigation was taken up.

The literature on this subject shows that an alkaloid has been found in 7 species of *Zanthoxylum*, but not in *X. acanthopodium*. Simonsen and Rau (2) isolated an alkaloid from the bark of another species, *Z. budrunga*, which also grows in the Darjeeling district but no work appears to have been done with its fruits. There is, however, no mention of the characteristic tingling sensation produced in the mouth by the fruits of *Z. acanthopodium* or of any other species so far studied chemically.

EXPERIMENTAL

Fruits and seeds.—The whole green fruits were crushed and made into a paste with anhydrous sodium sulphate and the mass was then extracted with petroleum ether. The essential oil was then separated from the extract by steam distillation after removal of the solvent. The oil obtained in the distillate was dried over anhydrous calcium chloride.

The yield of the dry essential oil was about 2.3 per cent. It is interesting to note that the yield from decomposed or sun-dried fruits was practically nothing. The interval between the collection of fruits at Darjeeling and their receipt at Calcutta was usually 3 days and during this period about 25 to 33 per cent. of the green fruits became dark brown and showed signs of decomposition in spite of the presence of an essential oil which was believed to have an antiseptic property. The change in the colour of the fruit from green to dark brown was accompanied by a perceptible change in its characteristic aromatic odour. Those showing signs of complete decomposition practically ceased to emit any aromatic smell, and on extraction they yielded only very small amounts of the essential oil. The drying of the fruit in the sun produced a similar change, and the essential oil disappeared almost completely. It appears that the essential oil undergoes a chemical change during the process of decomposition of the fruit.

The essential oil is colourless and transparent, with a specific gravity of 0.7679 (at 4°C) and a refractive index of 1.4567 at 40°C and 1.4505 at 26°C. It gives a bluish flourescence under the ultra-violet rays and has got an agreeable aromatic odour resembling, to a certain extent, that of eucalyptus oil.

Steam distillation of the extract mentioned above left a *non-volatile residue*, which was a brown resinous substance mixed with chlorophyl. It was found to be an oleo-resin, having a peculiar disagreeable smell. It was sticky and on keeping became almost solid and dark brown in colour, and also lost the intensity of its smell.

The fruits, both fresh and dried, were further analysed by the Stas-Otto process for the detection of an alkaloid or glucoside, if any, but nothing could be detected. This confirms the findings of Simonsen and Rao (*loc. cit.*) who also failed to detect any alkaloid in the seed.

Leaves.—The leaves and small twigs of Z. *acanthopodium* were also submitted to analysis but nothing except a trace of the essential oil could be isolated.

THE PHYSIOLOGICAL AND TOXIC EFFECTS

The essential oil when applied to the tongue produced a slight tingling sensation which lasted for a few minutes only. The resin, on the other hand, produced more well-marked tingling and smarting sensations which extended all over the mucous surface of the mouth and persisted for about two hours and were of the same intensity as that produced by the green fruits described before. It is, therefore, evident that this resinous substance is responsible for the physiological effects produced by fruits of *acanthopodium*. The irritant principles usually found in such plant products is a complex amide (Henry, 4) and the resinous substance was also found to contain an amide resembling *fagaramide* or *isobutyl-amide* of piperonyl acrylic acid which was isolated (Wehmer, 3) from Z *senegalense* DC, a species which does not grow in this country.

Both the essential oil and the resin do not appear to possess any property, sufficiently toxic to cause fatal poisoning. The administration of these substances to experimental animals did not produce any signs or symptoms of poisoning. A dose of 2 g. of the resinous substance administered to a kitten weighing about $2\frac{1}{2}$ kilos produced no untoward effects except some signs of local irritation in the mouth. This shows that the fruit does not contain any poisonous substance which is likely to cause death as claimed by the local people who, however, take it for its alleged medicinal properties.

The alcoholic and aqueous extracts of the fruits after separation of the essential oil and the resinous substances were also tried on cats but found absolutely harmless.

It may be noted in this connection that the fruits of Z. *budrunga* (mistaken for *acanthopodium* by our suppliers at Darjeeling and sent to us for our investigation) when submitted to the same methods of analysis, yielded also an essential oil and a resinous substance which was, however, found to differ considerably in its physiological properties from the resin obtained from the *acanthopodium* fruit. It produced only a mild and transient tingling sensation on the tongue. The *budrunga* fruit also differs morphologically from the other one in having a

smooth skin without any tubercles and its smell is exactly like that of the lemon-scented eucalyptus (*E. Citriodora*) and it produces a faint, almost imperceptible, tingling sensation when chewed. No alkaloid or glucoside could be detected in these fruits as well.

SUMMARY

The fruits of *Z. acanthopodium* contains a resinous substance which produces an intense tingling sensation in the mouth. The irritant principle present in the resin and responsible for the characteristic sensation is an amide, possibly fagaramide.

There is no poisonous principle in the fruit as to cause fatal poisoning. The belief among the hill people that the fruits are poisonous is therefore erroneous.

The fruit does not contain any alkaloid or glucoside and thus it resembles *Z. budrunga* which too contains no alkaloid. The latter, however, does not produce the characteristic tingling in the mouth.

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CATAPHORETIC PURIFICATION OF BACTERIOPHAGE

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Much has been said about the therapy of bacteriophage, both for and against its results, but it has not yet been possible to determine which of the components of the phage filtrate is responsible for the observed effects because bacteriophage filtrate usually comprises the phage corpuscles, bacterial substances (proteins, polysaccharides, enzymes, etc.) and the medium proteins. Many attempts have been made to get the bacteriophage corpuscles in a more or less pure condition.

The attempts of Arnold and Weiss (1) may be mentioned who tried to precipitate out the bacterial proteins by salt action but this process has not been attended with a large measure of success. Now-a-days the electrical behaviour of biologically active substances has attracted much attention. Von Angerer (2) reported that in an electrical field the lytic principle migrates towards the anode while Koch (3) using a similar technique reported migration towards the cathode. The above workers were not particular about the pH of the solution during cataphoresis. The pH is a very important factor during filtration and it has been found that a highly potent phage, if made acidic and filtered through porcelain candle, loses almost all the activity because in acid medium the phage corpuscles get adsorbed on the surface of the porcelain filter. In studies on the cataphoresis of phage particles, selection of dialysing media, diffusion and endosmotic effects are also to be considered and, if not properly controlled, they will lead to erroneous results. Todd (4) by means of cataphoretic studies found that Shiga bacteriophage carries a negative charge between pH 3.6 and 7.6. Kreuger, Ritter and Smith (5) reported that several races of coliphage carry a negative charge between pH 6.1 and 3.3 and flexner phage is negative between pH 5.4 and 9.3 and typhoid phage between pH 4.9 and 9.3. In the following experiments a simple cataphoretic method has been adopted for the preparation of bacteriophage free from proteins. This arrangement was designed and used by Ghose and De (6). The following bacteriophages have been used :—

Bact. Shigæ, Bact. flexneri, Bact. typhosum, Bact. coli, Bact. paratyphosum A and B and Staphylococcus aureus. The cataphoretic cell which was used is as follows :—
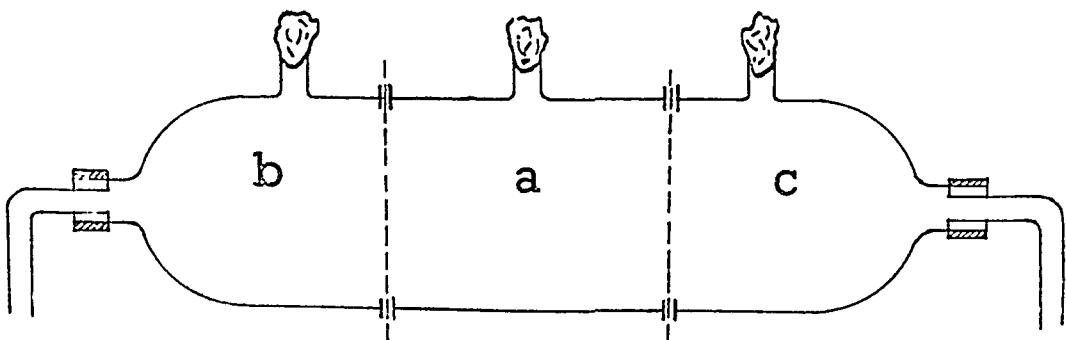
(*a*), (*b*) and (*c*) are three glass cells which are fixed tightly by pressure screws working at the ends. A cellophane membrane is placed between the two junctions of the cells. After assembling and fixing the cells it is tested for any leakage by pouring distilled water into (*a*) and keeping it for 12 hours ; ordinary phage filtrates adjusted to pH 7.2-7.4 are poured into the cell (*a*) and the side cells (*b*) and (*c*) are filled up with normal saline adjusted to the same pH as that of the cell (*a*). They are then electrically connected through a milli-ammeter and agar-NaCl tubes (NaCl 1% and Agar Agar 2%). The whole process is carried out

inside a refrigerator at 10°C or below and the current is maintained below 16 milliamps. After 5 hours the contents of the cathode and anode compartments are taken out, filtered aseptically through Pasteur Chamberland candle and the phage corpuscles are estimated by d'Herelle's dilution method (Roy (7)). The results are given in the following table.

Potency as corpuscles per cc.

	Before electro-dialysis.	After electro-dialysis.	
		Cathode cell	Anode cell.
Bact. shigæ	10^9	nil.	10^5
Bact. flexner	10^8	,,	10^5
Bact. typhosum (Rawlings)	10^5	,,	10^4
Bact. coli	10^6	,,	10^5
Bact. typhosum (0901)	10^7	,,	10^4
Bact. paratyphosum A.	10^7	,,	10^5
Bact. paratyphosum B.	10^6	,,	10^5
Staphylococcus aureus	10^{10}	,,	10^5

Cellophane membrane No. 400 was used as a dialysing agent. The dialysed filtrates of bacteriophage samples were water clear and gave negative tests with biuret and Millon's reagents and repeated injections of these dialysed products into rabbits did not give rise to any measurable titre of bacterial antibodies; rather, they produced a high titre of anti-phage bodies which has been noted by earlier workers.

SUMMARY

Filtrates of Bacteriophages adjusted at pH 7.2-7.4 were subjected to cataphoresis in a multi-chambered cell and it has been found that all the phages at the stated pHs migrate to the anode and further, their purity is increased.

6

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STUDIES ON THE SOMATIC ANTIGEN OF BACT. TYPHOSUM
PART I. ISOLATION AND TESTS.

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The typhoid bacillus being a flagellate has naturally two types of antigens as has been previously classified by Weil and Felix (1), the flagellar antigen being called H-antigen (Hauch) and the body or somatic antigen being called the O-antigen (Ohne hauch). Very recently another antigen—the virulence antigen classified as the Vi-antigen has been identified by Felix and his co-workers (2). The H-antigen is characteristic of all the motile species and the O-antigen is identical with the main bacillary body and is the same as the "endotoxin" because the toxic elements of the typhoid bacillus are mainly associated with it. This antigen has been the subject matter of our study. A strain of typhoid bacillus particularly rich in somatic antigen and almost free from other antigens was selected ; care has been taken to grow the organism in a suitable medium and to maintain it in smooth form. Some workers have preferred synthetic or semi-synthetic medium but the defect with such media is that the growth optimum for the organism is not fulfilled hence it is probable that the bulk yield of bacillary body as well as its antigen content will be proportionately diminished and moreover these variations may enhance the dissociation of the smooth to the rough phase. The use of solid media (with agar agar) was particularly avoided because the agar would come along with the bacillary body and this is very difficult to remove. Further it would give erroneous results in chemical components because agar agar is itself a complex polysaccharide composed mainly of galactose units which has been thoroughly worked out by Miles and Pirie (3) and Freeman, Challinor and Wilson (4).

The extraction of the antigen from the organism by drastic methods of tri-chloroacetic acid extraction used by Boivin *et al* (5) tryptic digestion method used by Raistrick and Topley (6) or extraction with solvents as carried out by Morgan (7) were avoided because of apprehension of any chemical or functional degradation of the antigen and therefore the method of extraction with normal saline at the *p*H identical with the natural system was adopted.

The following medium was used for the culture of the organism.

Peptone (Difco)	...	...	... 5.0 g.
Tryptone (Difco)	...	...	... 5.0 g.
Sodium chloride	...	...	... 5.0 g.
Dipotassium phosphate	...	...	... 1.0 g.
Distilled water	...	...	... 1000 cc.

The medium was prepared in a 2-litre flask and the *p*H was adjusted to 7.4 and sterilised in an autoclave at 15 lbs. pressure for ten minutes. It was filtered through paper and the *p*H was checked ; it was then resterilised at 15 lbs. pressure for 15 minutes and when the medium was cold, 10 cc. of a sterile solution of 20 p.c. glucose in distilled water were added aseptically with the help of a dry sterile pipette and the medium was kept for 3 days to check any contamination. In the meantime, the inoculum was prepared by transferring a loopful from a smooth colony of the strain 'ogoi' into 25 cc. of the liquid medium (of the same composition as stated above) ; the whole of it was used as the inoculum for 1 litre culture. 20 litres of culture formed one batch and after inoculation they were incubated at 37°C. for three days and then 5 cc. of glacial acetic acid were added to each litre of culture and they were kept inside a refrigerator overnight. In the meantime other batches were being worked up. To each of the chilled acidified cultures 1000 cc. of chilled acetone were added and after thorough mixing it was kept in the refrigerator for another two days ; the supernatant was then carefully drawn off and the residue was recovered by quick centrifugation. The combined sediments from the 20 litre batch were taken up in 500 cc. normal saline at *p*H 7.4 and shaken with glass beads in a mechanical shaker for 3 hours and again kept in the cold overnight ; the supernatant was drawn off and the residue was extracted by two further 250 cc. portions of normal saline at *p*H 7.4. The saline supernatants were mixed, filtered through Seitz filter to free the solution from bacillary bodies and again chilled in the refrigerator ; the solution was again treated with an equal volume of cold acetone and after 24 hours the supernatant was drawn off, the precipitate was quickly centrifuged and kept reserved. The precipitates from two other batches worked up according to this procedure were mixed with it ; further purification was started with this combined partially purified product from 60 litres of culture. The mixed residue was taken up in cold distilled water at *p*H 7.4 and again treated with an equal volume of cold acetone ; the precipitate was then shaken repeatedly by 100, 100, 50 and 50 cc.

portions of half-saturated ammonium sulphate solution at pH 7.4 and the filtrate was dialysed in a cellophane bag in the cold. The dialysed solution was again treated with an equal volume of cold acetone. The precipitate was taken up in the least amount of distilled water and finally precipitated by adding to a chilled solution inside a refrigerator thrice its volume of a chilled mixture of absolute alcohol and ether (3 : 1) and drying the precipitate *in vacuo*. The yield was approximately 1.2 g. from the 60 litres of medium used.

Chemical tests: Qualitative tests were carried out with a 0.2% solution of the substance in distilled water.

Xanthoproteic test	Positive (faintly)
Millons'	Negative.
Biuret	Positive (faintly).
Trichloroacetic acid 20%	No precipitate.
Esbach's reagent	No precipitate.
Hopkins -Cole	Negative.
Tollen's phloroglucinol	Negative.
Bials Orcinol-Hcl	Negative.
Acid permanganate	Very slowly reduced in cold.
Molisch's test	Strongly positive.
Fehlings	Negative.

On heating the substance in $2N$ hydrochloric acid in boiling water for 3 hours it reduced Fehling's solution and formed an osazone. The substance on sodium fusion gave no test for sulphur. On heating a solution of the substance in .1% acetic acid an insoluble fraction began to precipitate out which was mainly of protein in nature and the filtrate showed more markedly the presence of polysaccharide which as well the original substance gave precipitin reaction with pure O-antiserum. The average lethal dose of the substance for mice between 15-18 gms. was 0.25 mg. Immunising experiments as done by Topley *et al* (8) showed that the substance possesses a high degree of immunising capacity comparable with that of bacterial vaccines.

Further work on its chemical and antigenic analysis is in progress.

SUMMARY

A toxic antigen of typhoid bacillus identical with O-antigen has been isolated. The substance as shown by chemical tests is free from sulphur, tryptophan and pentose residues.

Polysaccharide liberated after dilute acetic acid treatment behaves as O-hapten.

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ROLE OF IRON IN ANÆMIA DURING SCURVY

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It has long been recognised that scurvy is accompanied by a fall in the hæmoglobin level of the blood. How far the anæmia is the result of derangement of iron metabolism due to the specific lack of ascorbic acid is however not yet definitely known.

Parson (1) observed that the effect of vitamin C upon the maturation of red cells parallels that of thyroxine. According to him, as 'a result of an inadequate intake of this vitamin, anæmia, both of orthochromic normocytic type and of the macrocytic type results. Both the forms of anæmia were cured by the administration of ascorbic acid without any other alteration of the diet and without giving any drug. Such results seem to furnish considerable evidence that vitamin C is necessary for the normal maturation of red cells, yet there were cases where severe scurvy occurred without any sign of anæmia. In seven cases of infantile scurvy out of fourteen, he observed severe anæmia which was cured by ascorbic acid but in the rest no sign of anæmia was found—a fact that was difficult to explain.

Loznes (2) observed that in four out of five patients with moderate anæmia and with complete absence of ascorbic acid in the blood plasma, hæmoglobin regeneration occurred spontaneously or in response to iron therapy alone. In three of the patients, administration of ascorbic acid after the initial period of hæmoglobin regeneration neither caused reticulocytosis nor increased the speed of further hæmoglobin increase.

Investigations of D'Alessandra (3) have shown that ascorbic acid injected subcutaneously into normal rabbits increases slightly the number of R.B.C., while that of W.B.C. remains the same. When ascorbic acid is injected into rabbits which have been bled to produce a secondary anæmia, the increase of red cells

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becomes greater, increase being accompanied by the appearance of reticulocytes and nucleated red cells.

Alt, Chinn and Farmer (4), on comparing the plasma ascorbic acid in 14 patients with achlorhydria, associated with pernicious or iron-deficiency anæmia in remission or relapse with 24 controls, observed that with diet adequate in vitamin C, the ascorbic acid is significantly decreased in cases of pernicious anæmia but not in the iron-deficiency group. With diets inadequate in vitamin C, the plasma ascorbic acid is significantly decreased in both the groups.

Stacy and Chew (5) believed that the anæmia during scurvy is not due to the lack of iron but do to the retarded erythropoiesis. The oral administration of iron was shown to be ineffective in alleviating the anæmia of human scurvy (Mettier, 6).

In a series of experiments with guineapigs in which scurvy was produced by feeding the diet of Harris and his co-workers (7), McFarlane (8) observed severe anæmia. During the recovery which followed the injection of 3 mg. of ascorbic acid daily, the hæmoglobin and red cell count returned to the normal level. When next, the ascorbic acid was withheld and each guineapig received instead of a daily intraperitoneal injection of 3.2 mg. of Fe″ or Fe″, in the form of Mohr's salt or ferric chloride, the animals again lost weight. All became anæmic and died of scurvy within 12 days. When 6 guineapigs were fed the same diet and received daily injections of 3.2 mg. of Fe″ as Mohr's salt, severe scurvy, marked by inanition, rapid loss in weight and the development of a pronounced secondary anæmia, was produced in all the animals.

We have reported earlier (9) that with the progress of scurvy there is a progressive diminution in the blood hæmoglobin level of guineapigs which is restored almost to the normal level after supplementing the scorbutic diet with ascorbic acid. The lowered hæmoglobin level does not appear to arise merely from a lowered food-intake and therefore a lowered iron-intake which occurs in scurvy. This is clear from the fact that when animals, receiving ascorbic acid, were given restricted amounts of diet equal to those consumed voluntarily by corresponding guineapigs on a scorbutic ration during the previous 24 hours, thus limiting their intake of iron, those animals nevertheless showed a normal blood hæmoglobin level.

The anæmia in scurvy, therefore, appears to be of more fundamental origin. The question arises whether the absence of vitamin C diminishes the absorption of iron in the body and its retention for blood formation. This question has been investigated in the present work.

Along with this investigation, the change in the plasma-iron content with the progress of scurvy has been studied, because, the iron content of the plasma, though it is very small in comparison with the total body iron, probably plays a very important role regarding the transport of iron for hæmoglobin formation and regeneration (Tompsett 10 ; Barkan 11).

Barkan (11) observed an increase in the iron content of the plasma when the whole blood was allowed to. stand. He (12) presented the hypothesis that this

increase had its source, not in hæmoglobin itself, but in partial degradation products of hæmoglobin, normally present in the blood (the "easily split-off" blood iron). He, therefore, designated this as *pseudo-hæmoglobin*.

Tompset (13) has shown that when the plasma is incubated with HCl at 37°C the iron liberated is in the ferrous state. He also observed that certain food-stuffs are capable of reducing ferric iron to the ferrous state, particularly in an acid medium.

Now, the problem is, which dietary factor causes this reduction? If it is due to the ascorbic acid and if the reduction of ferric iron is necessary at any stage in the mechanism of blood formation, it is possible that there might be some physiological relationship between the vitamin C content of the blood and plasma iron.

EXPERIMENTAL

Twenty healthy male guineapigs, all having approximately the same weight were fed on the same normal diet of green grass and germinating gram for two weeks prior to the actual experiment. The animals were then kept in individual aluminium cages with screened bottoms. They were fed on scorbutic diet. Ten of the animals received in addition to the scorbutic diet, 2 mg. of ascorbic acid per head per day and thus treated as positive controls. Prior to the beginning of the experiment, the body-weights were taken and hæmoglobin and erythrocyte concentrations of the blood of the animals determined by the usual method described earlier (14). The food consumption and bodyweights of the animals were recorded and fæces were carefully collected after every four days. All possible precautions were taken to avoid contamination of the food with iron. The fæces were dried at 105°C, adhering food, if any, was separated, weighed and finally powdered in a glass mortar. Weighed quantities of the fæces were taken and the total available iron were determined by the methods described elsewhere. When acute scurvy developed in the negative controls, blood hæmoglobin and erythrocyte concentrations of all the animals were again determined and in the positive control and the total iron content of blood in each individual animal was determined by ashing. The results are shown in Tables I, II and III. The total and available iron in the scorbutic ration were determined as usual.

Determination of Plasma Iron :

Twelve healthy male guineapigs all having approximately the same weight were divided into 2 equal groups. All the animals were kept on scorbutic diet but the animals of the 2nd group, which served as positive control, received in addition to the scorbutic diet, 2.0 mg. of ascorbic acid per animal per day. After a period of 24 days, when acute scurvy developed in the animals of the 1st group, 5.0 cc. of blood were drawn out from the heart of each animal of both the groups. Plasma was separated from the whole blood by centrifuging. Two cc. of plasma were taken in each case and the plasma-iron was determined by Hill's modified method (16). The results are given in Table IV.

TABLE I

Initial and final blood hæmoglobin and erythrocyte levels of guineapigs kept on scorbutic diet only and those receiving 2 mg. of ascorbic acid as a suplement per animal per day.

Animal No.	Initial		Final		Supplement.
	Hb in %	R.B.C. in millions	Hb in %	R.B.C. in millions	
1	85	4.5	69	3.2	
2	103	4.4	65	3.0	
3	92	3.8	71	3.1	
4	90	4.5	67	3.3	
5	90	4.6	68	3.4	No supplement.
6	101	4.7	60	2.7	
7	110	4.1	72	2.6	
8	116	5.1	83	3.2	
9	96	4.0	57	2.4	
10	108	4.5	62	3.2	

Animal No. 11 died in course of the experiment from unknown causes.

12	91	4.2	92	3.9	
13	100	5.2	87	4.2	
14	85	4.6	91	4.4	
15	100	4.6	95	3.5	2.0 mg. of ascor-
16	103	4.6	102	4.1	bic acid per
17	102	4.1	96	4.1	animal per day.
18	88	3.8	98	4.0	
19	114	5.3	98	3.8	
20	98	4.3	100	4.5	

TABLE II
Iron content of the livers of animals expressed in mg.

Animal No.	Weight of whole liver in g.	Percentage of iron in the liver.	Iron in whole liver.	Supplement.
1	13	6.30	0.8 9	
2	11	11.20	1.232	
3	10	12.20	1.220	
4	9	14.00	1.260	No supplement.
5	12	6.00	0.730	
6	9.07	17.60	1.	
7	9.20	15.00	1.380	
8	6.10	17.60	1.680	
*9	—	—	—	
*10	—	—	—	
11	12	8.50	1.020	
12	11	6.90	0.770	
13	9	12.00	1.080	
14	10	10.30	1.120	2.0 mg. of ascorbic
*15	8.5	16	1	acid per animal
16	7.65	17.60	1.210	per day.
17	8.10	1 .00	1.050	
18	8.50	18.00	1.530	
19	4.70	15.00	0.719	

*Animals Nos. 9, 10 and 20 died in course of the experiment.

TABLE III
' *Iron values are given in mg.*

'A' signifies the intake of food iron, 'B' the total iron excreted and 'C' the total available iron excreted.

Animal No.		Intake and excretion of iron for six successive 4-day periods.						Total intake and excretion of iron in 24 days.	Iron balance.	Supplement.
		1	2	3	4	5	6			
1	A	6.5	13.2	18.3	19.5	6.0	3.4	68.9	−10.2	
	B	11.0	11.0	21.0	20.0	7.1	4.0	77.1		
	C	9.2	13.0	17.6	16.3	5.3	3.2	61.0		
2	A	5.6	11.6	20.3	20.0	5.1	4.6	70.2		
	B	6.8	15.2	20.0	21.2	6.9	3.9	73.9	−3.7	No supple-
	C	5.3	13.6	19.3	18.6	6.3	1.1	67.2		ment.
3	A	6.2	15.0	20.2	18.6	7.2	5.0	72.2		
	B	5.9	16.3	20.6	17.0	7.7	4.9	72.4	−0.2	
	C	5.3	14.5	17.0	16.6	6.0	3.0	62.4		
4	A	6.5	13.6	14.2	14.1	5.7	2.6	56.7		
	B	4.6	14.3	15.9	15.0	6.3	3.6	59.7	−3.0	
	C	4.2	12.2	14.1	11.0	4.6	2.4	51.5		

Animal No.		Intake and excretion of iron for six successive 4-day periods.						Total intake and excretion of iron in 24 days.	Iron balance.	Supplement.
		1	2	3	4	5	6			
5	A	6.1	13.8	18.2	19.0	6.7	5.3	69.1		
	B	5.6	14.3	17.6	17.8	7.6	4.8	67.7	2.4	
	C	5.2	10.2	15.2	15.4	6.2	4.3	56.5		
6	A	8.2	13.4	14.8	16.6	10,6	8.0	71.6		
	B	10.9	11.2	25.0	12.9	12.3	14.2	86.5	−14.9	
	C	5.5	7.5	14.1	10.0	8.3	6.1	51.5		
7	A	4.1	8.6	12.2	15.2	4.6	8.6	53.6		
	B	0.9	2.7	7.8	18.4	4.6	4.4	38.8	14.8	No supplement.
	C	0.7	2.6	7.8	9.5	3.2	3.1	26.9		
8	A	13.0	19.8	19.0	19.4	8.0	12.6	91.8		
	B	18.0	23.2	18.6	14.5	7.5	13.6	95.4	−3.6	
	C	5.7	14.7	13.3	10.3	5.8	11.2	61.0		
9	A	9.2	17.6	16.2	15.8	8.2	5.6	72.6		
	B	4.8	13.5	16.2	10.3	9.6	3.8	58.2	14.4	
	C	4.8	9.0	16.2	9.3	6.5	2.6	48.4		
10	A	11.2	17.2	13.4	10.8	10.2	8.6	71.4		
	B	5.8	22.3	10.1	16.4	8.4	9.0	72.0	−0.6	
	C	4.8	14.0	6.7	9.3	6.8	6.3	47.9		
11	A	9.2	15.1	20.2	19.0	17.0	16.3	96.8		
	B	7.3	14.2	19.3	18.0	15.1	14.3	88.2	8.6	
	C	7.2	12.0	16.6	16.3	14.6	13.8	80.5		
12	A	10.2	22.2	23.2	20.9	23.8	19.3	119.6		
	B	9.6	22.5	25.6	22.1	19.5	21.1	120.4	−0.8	
	C	8.8	20.0	23.2	20.1	18.2	18.8	109.1		
13	A	7.3	19.6	18.2	19.8	18.0	18.3	101.2		
	B	6.8	17.5	20.3	18.2	17.3	16.6	96.7	4.5	
	C	6.2	15.3	18.2	16.1	15.2	14.1	85.1		
14	A	6.8	15.6	18.3	21.2	19.8	19.6	101.3		2.0 mg. of ascorbic acid per animal per day.
	B	6.2	13.2	19.3	23.9	18.2	17.6	98.4	2.9	
	C	5.8	12.6	16.3	21.2	16.0	11.3	86.2		
15	A	11.2	17.6	13.6	10.2	13.4	16.8	82.8		
	B	10.50	26.50	11.70	11.00	12.50	11.20	86.1	−3.6	
	C	9.70	25.0	11.70	8.30	10.20	13.00	77.9		
16	A	6.0	12.6	13.8	14.50	11.60	13.8	72.3		
	B	3.10	10.90	14.00	16.30	10.30	10.7	65.3	7.0	
	C	2.30	6.90	13.4	10.3	8.3	8.3	49.5		
17	A	11.20	17.0	11.8	17.6	11.6	10.0	85.2		
	B	9.01	16.3	10.33	13.8	11.4	4.5	65.3	19.9	
	C	8.30	15.90	8.3	12.6	10.0	3.3	58.4		
18	A	10.2	15.2	18.0	19.6	17.2	15.6	95.8		
	B	7.3	9.9	15.6	19.1	17.9	13.8	83.6	12.2	
	C	5.3	8.8	12.9	13.2	15.4	10.4	66.0		
19	A	6.2	11.6	10.4	13.4	7.8	13.6	63.0		
	B	2.03	8.3	16.7	16.2	6.70	7.5	57.4	5.6	
	C	1.51	7.5	14.5	10.4	4.7	5.4	44.0		

TABLE IV

Plasma-iron content of animals, in mg. per 100 cc. of whole blood.

Plasma-iron of animals fed on green grass and germinated gram.	Plasma-iron of animals fed on scorbutic diet supplemented with 2.0 mg. of ascorbic acid per animal per day.	Plasma-iron of animals fed on scorbutic diet per 21 hours.
0.110	0.106	0.062
0.120	0.096	0.068
0.090	0.089	0.072
0.120	0.110	0.053
0.085	0.130	0.056
0.101	0.102	0.070

DISCUSSION

Table III indicates the iron balance of the animals on scorbutic diet and of those receiving vitamin C. A, B and C in Table III represent the intake of total food iron, total and available iron in the excreta respectively. As the iron excreted through the urine is very small in comparison with the total iron-intake the estimation of iron in the urine has not been carried out. With scorbutic animals, it has been found that in the last week of the experimental period, *i.e.*, just with the appearance of scurvy, there is a gradual decrease in the food-intake and thereby in the iron-intake. Although the majority of animals on scorbutic diet are found to be in negative balance with respect to iron (Table III), the amount is insignificant in comparison with the total intake and excretion of iron in course of 24 days and therefore the animals, even at the advanced stages of scurvy may be considered to be in iron balance. There is no significant difference in the liver iron of the two groups of animals. So long as the iron-balance is maintained, even inspite of reduced iron intake, the iron stored in the liver should have been sufficient to permit the hæmoglobin content of the blood to be maintained at the normal level during the short period of extreme inanition.

During advanced stages of scurvy, hæmorrhages occur upon slight injury. Hæmorrhage is the predominating feature of the disease and when it occurs internally, it is frequently the cause of death. Our purpose of estimating the available (non-hæmin) iron in the excreta was to see whether there was any internal hæmorrhage which might be the reason for anæmia during scurvy.. But from Table III it is clear that there is no significant variation in the available iron content and therefore in the hæmin-iron content (difference between the total and available iron) of the fæces of the guineapigs in normal, prescorbutic and scorbutic periods. The absence of this variation in the fæcal hemin of scorbutic animals would indicate either that there was no extensive internal hæmorrhage (which would be likely to lead to the increase in fæcal hemin) or that the hæmorrhagic hemin is stored in the body. In the latter case this hemin is apparently incapable of utilisation for blood formation as the anæmia produced in scurvy indicates.

however (8), reports potential danger of toxic action on kidneys by phosphotungstate and Bertram *et al* (9) point out the defects of "Cholosulin", the preparation of insulin and· desoxycholic acid. Synthalin (di-guanidine deca-methylene) the synthetic preparation of Frank (10) when taken orally was claimed to give excellent results. Further study indicated that it has a very definite toxic action not only on the kidneys but also on the liver.

The most notable advance in the insulin therapy has, in recent years, been made by Hagedorn and his collaborators (11) at Copenhagen. Protamin insulin (P.I.), as prepared by them, has a great beneficial effect of prolonging the action of insulin and thus reducing the injection to once a day from two or three times a day. Beecher and Krogh (12) observe that while insulin disappears from the tissue in about 45 minutes protamin insulin requires about 5 hours. A better achievement than this has also been reported by Scott and Fisher (13) who found that zinc, in concentration of 1%, greatly delays and prolongs the effect of protamin insulin on rabbits, and has also proved beneficial in cases of human diabetic patients. Introduction of Z.P. insulin has made it possible to treat a diabetic with fewer daily injections and smaller daily dose. But it is also not free from danger. Clark (14) points out that if hypoglycemia, is ·produced by injection of Z.P.I., it is very difficult to treat. According to Joslin (15) the diabetic story has not yet been complete and just when we think the end is approaching, a new chapter is written. He states " . . . The diabetic taking insulin is like a rapidly moving machine, which a slight swerve of the wheel will bring to disaster." He further adds "the treatment of the diabetic has not been simplified by insulin, but has been made complex and, temporarily, with protamin insulin even more so." Merchant (16) points out that along with the spread of civilisation there has also been a gradual and steady rise of the incidence of diabetes, and no method has been known at least to keep its incidence under proper control.

Isolation of a new antidiabetic ·compound from *Scoparia dulcis*, Linn., has recently been reported by the author (17) and named *amellin* for its specific property of causing relief in diabetes mellitus (18). This compound has been found to be highly effective by oral administration and superior to insulin in various respects. The present paper deals mainly with some biochemical properties of this compound.

Some observations in vivo

Physiological influence of amellin in reducing glycosuria and other associated troubles, has already been reported (*loc. cit.*). Details concerning some of the patients and results of clinical analyses of their urine samples are shown in the following table. Amellin was administered in the daily dose of 15 to 20 mg. only along with some calcium salt which caused relief in all the cases within a fortnights' time. Results of detailed investigations will be communicated in the parts following.

TABLE I.

Pt. No.	Duration of disease and historical notes.	Age years.	Body Wt. lbs.	Average daily-calorie intake (approximate).	Calorie recommended by Dreyer (19).	g. of reducing sugar excreted in 100 cc. of urine (12 hours' sample). On days.									Remarks.
						0	4	6	7	9	12	16	19	40	
X.	Suffering for 1 yrs., but could not afford to pay for costly treatments.	45	112	2200	1509	2.270	0.630	...	...	0.080	...	0.060	...	Nil.	
Y.	Detected only five months back, daily dose of 20 units of insulin were being taken.	37	161	2000	1656	3.400	2.000	0.080	...	...	...	...	...	...	Amellin was given on the 4th day when insulin was stopped.
Z.	Suffering for about 1 year, used insulin for 3 months prior to this treatment.	15	150	2800	1550	3.100	...	...	2.000	1.430	0.910	0.580	Nil.	...	

Studies in vitro .

-Effect of amellin on the oxidation of glucose in presence of human plasma and physiological saline solution.

Lundsgaard and Holboll (20) observed that when insulin and muscle tissue were added to solutions of ordinary glucose, there was a decrease in the optical rotatory power of the dialysate without any change in the reducing power. Insulin alone or muscle tissue alone could not produce this change. From this it was supposed that insulin would bring about only some sort of change in the glucose molecule so as to give rise to an isomer. They did not find any evidence that insulin (alone or with blood or muscle tissue) was able either to burn sugar or transform it into glycogen. Winter and Smith (21) have also observed similar effect on glucose by insulin, in presence of liver extract. Alteration of the optical property of the sugar solution was also suggestive of formation of γ-glucose in the resultant mixture. Other important findings of these authors (22) indicate, that the sugar in blood of normal animals is probably γ-glucose. It was therefore suggested that glucose must exist in the gamma form such that it can easily (*i*) synthesise glycogen in the liver or (*ii*) undergo oxidation by tissues.

It would be interesting, therefore, to see whether the new compound amellin can bring about some change in the reducing power of glucose and thus yield some explanatory note in causing sugar to be polymarised or metabolised in the system of diabetic patients.

EXPERIMENTAL

One cc. solution of amellin in physiological saline solution (about 10 mg.) was poured in a flat bottom-flask (25 cc. capacity) in which 5 cc. physiological saline solution had already been added and 1.0 cc. of plasma from human blood was added to this. Now 1. cc. of a very dilute glucose solution (containing about 1 mg. per cc.) was added to this and the mixture incubated for about half an hour in a thermostat at about 37°C. Sugar oxidised, if any, during the process was obtained by estimating the remnant sugar from the resulting mixture and from the original solutions of sugar, plasma and amellin individually, according to the micro-method of Shaffer and Hartmann (23) as modified by Stiles *et al* (24). The result was found out in terms of milligrams from the difference in titration value between the blank and the sample with the help of a chart supplied by them.

The result in the following tables will show how far a glucose solution can be oxidised by amellin with or without plasma from normal and diabetic individuals in presence of physiological saline solution at 37°. This is to be noted that amellin alone has the property of reducing copper sulphate solution. This may be either due to some ·trace of adhering ·impurity or to some group in the molecule.

TABLE II.

Effect of amellin on oxidising glucose in presence of plasma of normal human blood and physiological saline solution.

Temp. of the thermostat—37°

Solutions in the vessel.	.005 N. thio in cc. required.	Reducing sugar in terms of glucose in mgs.			Sugar lost or oxidised %	
		Calculated on the basis of direct addition.	Actually found.	Lost or oxidised during reaction.	Calculated.	True.
(1) Amellin 1 cc.						
(a) 10 mg. per cc.	4.2	...	0.631			
(b) 12 mg. per cc.	5.0	...	0.745			
(2) Glucose 1 cc.						
(a)	6.0	...	0.882			
(b)	5.7	...	0.837			
(3) Plasma 1 cc.						
(a)	8.3	...	1.186			
(b)	8.4	...	1.198			—
(4) Plasma 1 cc. and glucose 1 cc.						
(A) (from 2a & 3a)	13.3	2.068	1.813	0.255	...	12%
(B) (from 2b & 3b)	13.2	2.035	1.800	0.235	...	11%
(5) Amellin 1 cc. and glucose 1 cc.						
(A) (from 1a & 2a)	12.7	1.516	1.728	−0.212		
(B) (from 1b & 2b)	13.7	1.582	1.871	−0.289		
(6) Amellin 1 cc., and glucose 1 cc. and plasma 1 cc.						
(A) (from 1a, 2a & 3a)	14.0	2.702	1.913	0.789	30%	35% (from 3a & 5A)
(B) (from 1b, 2b & 3b)	12.4	2.780	1.687	1.083	39%	46% (from 3b & 5B)

TABLE III.

Effect of amellin on oxidising glucose in presence of plasma of blood from fasting diabetic patients and physiological saline solution.

Temp. of the thermostat—37°

Solution in the vessel.	.005 N. thio in cc. required.	Reducing sugar in terms of glucose in mgs.			Sugar lost or oxidised %	
		Calculated.	Found.	Lost or oxidised.	Calculated.	True.
(1) Amellin 1 cc. 10 mg. per cc.	3.9	...	0.583			
(2) Glucose 1 cc.	6.0	...	0.882			
(3) Plasma 1 cc.	5.9	...	0.868			
(4) Plasma 1 cc. and glucose 1 cc. (2 & 3)	12.3	1.750	1.674	0.076	5%	...
(5) Amellin 1 cc. and glucose 1 cc. (1 & 2)	12.4	1.465	1.687	−0.222		
(6) Amellin 1 cc., Glucose 1 cc. and plasma 1 cc. (1, 2 & 3)	13.9	2.333	1.899	0.434	18%	25% (considering 3 & 5).

It is evident from the Tables II & III that the degree of oxidation of glucose by amellin in presence of diabetic plasma is lower than that with normal plasma. But yet the results indicate very nicely the superiority of amellin over insulin in causing direct oxidation or metabolism of reducing sugar present in the system.

POLARIMETRIC STUDIES

In order to gain some idea about the intermediate changes in the reaction it was contemplated to see whether there was any difference between the optical rotatory power and the reducing power of the dialysate after allowing a glucose solution to react with amellin and plasma, blood or muscle tissue for a particular period. This was done according to Lundsgaard and Holboll (*loc. cit*) with slight modification as follows:—

The experiments were performed in 200 cc. reagent bottles. About 20 mg. of amellin and 2 g. of glucose dissolved in measured quantity of physiological saline solution, were introduced in the bottle no.I. 10 cc. freshly drawn blood from normal human subject or 15 g. of muscle tissue from rats excised immediately were then introduced in it and the total volume made 100 cc. with

physiological saline solution instead of .9% NaCl solution as used by previous workers. The bottle was then introduced in a thermostat at 37° and shaken frequently. Samples of mixtures were withdrawn (20 cc. at a time) initially and after different intervals of time, introduced in parchment paper-bag dipped in 40 cc. physiological saline solution in a beaker, and dialyzed at room temperature, each for about 2 hours and a half. The reducing power of the dialysate was determined according to the method of Shaffer and Hartmann (lot. cit) and the rotatory power was determined by the polarimeter in the usual way. 12 readings were taken each time and the average figure was taken. The greatest variation in numerous controls did not exceed .02°.

A parallel experiment was also made in bottle No.II. with everything in the equal amount except that it contained no amellin. Preliminary results obtained so far regarding the effect of amellin on glucose, when blood or muscle tissue is added, does not indicate the possibility of transformation of ordinary glucose (α, β-glucose) into the γ form which has lower rotatory power. There is no appreciable change in the rotatory power of the dialysate even after two or three hours' reaction. Detailed studies, however, in this line showing influence of time, temperature and quantity of added blood or muscle tissue on the difference in glucose equivalents between reducing and rotatory power of the dialysate, will be reported in a later communication.

From the observations made so far the following possibilities of the activity of amellin can be suggested:—

(i) Amellin may be a new type of chemical compound (antidiabetic principle), the deficiency of which causes faulty oxidation of glucose in the system and thus plays the part of accelerating the process of internal oxidation.

(ii) It may counteract the diabetogenic effect of anterior pituitary, adrenals and thyroid hormones.

(iii) Degenerated β-cells of the islands of Langerhans, may be regenerated by this, thus causing greater secretion of internal insulin.

(iv) This may destroy or neutralise the effect of such inhibitors to insulin or toxins as might be formed and absorbed through insufficiency of oxidation of intermediate metabolism products.

SUMMARY

1. Amellin, which has been found to cause relief in glycosuria in diabetics, can also oxidise glucose in vitro, in presence of plasma of human blood, and physiological saline solution.

2. There is also slight oxidation of glucose even in absence of blood or plasma.

3. In presence of diabetic plasma, however, the degree of oxidation of glucose brought about by amellin is smaller than that with normal plasma.

4. Preliminary polarimetric studies do not indicate the possibility of transformation of ordinary glucose into its γ form by amellin in presence of human blood or muscle tissue from rats.

ACKNOWLEDGEMENT

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INVESTIGATIONS ON THE NEW ANTIDIABETIC PRINCIPLE (AMELLIN) OCCURRING IN NATURE—PART II—ITS EFFECT ON GLYCOSURIA AND HYPERGLYCEMIA IN CASES OF HUMAN DIABETES

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An absolute or relative lack of insulin, since its discovery from the Islets of Langerhans (1), has been known to cause failure in· the regulation of blood sugar level and to bring about what is termed as hyperglycemia and glycosuria. But it was Mann (2) who for the first time established that the liver is the site of formation of blood sugar and the prime factor for maintenance of its normal level. Soskin (3) confirmed satisfactorily that the liver is the sole source for supply of the glucose in the blood. Roy (4) and Mukherjee (5) rightly pointed out that the liver is primarily · responsible for the onset of hyperglycemia. Roy and Mukherjee (6) further suggest that the first stage in the development of hyperglycemia is a condition of acidosis, which may result from excess intake of acid bodies or from faulty method of living specially on high protein and fat diets. They also hold that the hyperglycemia of diabetes, which has so long been considered as a pathological process, is nothing but a physiological response to certain metabolic defect. Lawrence (7) and Joslin (8) pointed out that action of insulin is only· limited to glycogen formation. But the idea regarding the sole activity of insulin in the restoration of glycogen and its utilisation in the system, has been revolutionized by the important findings of Dambrosi (9) and Lukens *et al* (10) who have shown that restoration of muscle glycogen after depletion through exercise is just as complete in depancreatized as in normal animals except that in the former (*i.e.* in absence of pancreas and hence insulin) it takes place at a comparatively lower rate. They also noticed that until certain level of blood sugar the depancreatized animal utilises less sugar than the normal ; but when the limit is exceeded, this difference disappears and the same rate of utilisation prevails. These observations may lead to the following suggestions:

(i) insulin acts just like an activator to some chemical reactions and .

(ii) in the animal system there are things other than insulin which may bring about carbohydrate metabolism, though at a considerably slower rate.

That insulin again requires activation, has been evident from the extensive studies by Himsworth (11). He observed that in human subjects, after insulin injection, there was a latent period before the fall in blood sugar commenced, and even a second dose, about thirteen minutes after the first one, showed the same type of late effect. On the basis of his findings he proposed the theory that the latent period is an expression of the activation of insulin. He went still further and suggested that insulin, as secreted by the pancreas, is physiologically inactive, and is activated by some other unknown factor which he termed as "Insulin Kinase", possibly produced in the liver. This theory has in recent years been strengthened by the observations of Subrahmanyan and his associates (12), who succeeded in isolating inactive insulin from beef pancreas but having all the physical and chemical properties of standard insulin. 'This latter view can also be substantiated from the statement of Clark (13) who pointed out that in some cases of diabetes there was no visible lésion of the islet tissues and the disease might be due to the excessive activity of the anterior pituitary. Thus inspite of the fact that insulin is secreted in abundance in the system its action was hindered by anterior pituitary hormone which possibly has the power of neutralising or destroying the supposed activator. An additional support to this view can also be had from the recent findings of Roy (4) and Roy and Mukherjee (14). They have shown that the tissues of a diabetic can oxidise sugars if present in a suitable form and gluconeogenesis—abnormal production of sugars from proteids and fats—can also be lessened to a great extent only by intravenous injection of glucose to the patients after fasting for about four days.

That the deficiency of some unknown factor is in some cases, responsible for the occurrence of diabetes mellitus, has also been clear from the recent work of Himsworth (15). He divides diabetes in two classes ;

(i) insulin sensitive and (ii) insulin insensitive,

the latter type suffering entirely or partly from the deficiency of the unknown factor. Similar idea regarding the presence of an insulinotropic principle, in the duodenum, was also postulated by Langhton and Macallum (16) and La Barre and his colleagues (17). Amellin, the new antidiabetic compound has recently been suggested by Nath (18, a, b) to be related to this unknown factor, deficiency of which might cause disturbance in the carbohydrate metabolism thus leading to onset of diabetes mellitus. These findings, it is demanded, may, as postulated recently by Merchant (19), completely metamorphosize all previous ideas about diabetes and may be of some relief at least to the cause of suffering humanity.

The present paper deals with details regarding the physiological effects of this compound on human diabetic patients specially in reducing hyperglycemia and glycosuria. It will be quite evident from the tables, figures and statements of the patients themselves that apart from the relief of these symptoms, they observed marked improvement in general health accompanied with the relief in all other troubles associated with diabetes.

EXPERIMENTAL

Records of clinical results of fifteen diabetics of different age, sex, religion and profession, will be shown in this part.

Table I gives some informations regarding the patients.

Diet :

Regulation of diet has long been recommended as one of the important factors in the treatment of diabetes. Basal requirement (calories) as suggested by Dreyer (20) for body weight as high as 150 lbs., does not exceed 1600 cals. for ages about 30 years. Glark (*loc. cit.*) suggests taking of 120 g. of carbohydrate which renders the incidence of ketosis unlikely and supplies a total calorie of 480 only, and Joslin (21) recommended a basic diabetic diet for an adult to comprise of the followings:

Carbohydrate	150 g.	600 calories
Protein	70 g.	285 ,,
Fat	80 g.	720 ,,
Total		1600 calories

According to Beaumont and Dodds (22) maximum calories allowed are 15 cals. per pound of body weight (Table II).

It would be better for us also if we could have regulated the diets of our patients accordingly. But as all the patients resided in their respective homes, we allowed them to go on with the diets with which they were habituated. Only they were advised to keep up the uniformity of diet which helped us in calculating the amount of daily calorie intake from the charts.

Table II will indicate the total quantity of food intake of the individual patients in 24 hours and the calculated daily calorie intake. It will be evident from this table that there is not a single diet chart in which the total quantity of carbohydrate comes lower than 206 g., the highest limit being 545 g., which corresponds to 2234.5 calories (from carbohydrate alone) ; but inspite of this, highly satisfactory results have been achieved.

Basal Requirements :

Basal requirements (calories) as recommended by authorities Dreyer (20) and Beaumont and Dodds (22) and the calorie allowed by us are shown in Table II. In many cases, as will be seen from the table, the calories are far greater than those recommended during insulin treatment. Thus the question of underfeeding the patients and thus making them aglycosuric by restriction of diets and dietetics does not arise at all.

TABLE I.

Patient No., Sex, Age.			Religion.	Profession.	Duration of disease.	Medicine used before.	When our treatment begun.	24 hrs' Urine sugar p.c.	
								Initial.	Present.
1	Male	56	Hinduism	Medical officer S.A.S.	6 years	None	29.7.41	5.7	Nil.
2	"	42	"	Businessman	3 "	Insulin	5.8.41	3.27	Nil.
3	Female	48	"	School teacher	6 "	Oralin	6.9.41	2.5	Nil.
4	Male	48	"		6 months	None	15.9.41	5.4	Nil.
5	"	75	"	Rtd. Government officer	25 years	Insulin for about 16 years	4.12.41	1.9	Nil.
6	"	60	"	Zamindar	10-12 years	Insulin (60 units daily) and other medicine	22.12.41	3.57	
7	"	33	"	Money-lender	4 years	Opium and Jambulin	8.1.42	2.5	Nil.
8	"	43	Islam	Clerk in Government office	9 "	Pancrepatin	23.2.42	4.35	2.8 on the 80th day
9	Female	50	Hinduism		...	None	23.2.42	2.5	Nil.
10	Male	62	Islam	Rtd. Income-tax officer	10 years	None	9.3.42	3.3	0.06 on the 85th day
11	"	49	Hinduism		6 "	None	11.3.42	0.9 (After 3 hrs. taking a mixed meal)	Nil. (under the same condition)
12	"	49	"	Merchant	9 months	Insulin (60-80 units daily)	11.3.42	6.25	4.3 on the 38th day
13	"	69	"	Rtd. officer	8 years	Insulin 20 units daily	18.3.42	7.13	Nil.
14	"	50	"	Medical officer of some charitable dispensary	2 "	Strict regulation of diet alone	19.3.42	Nil.	Nil.
15	"	59	Islam	Rtd. Government officer	7 "	Insulin	11.4.42	1.8	0.24 on the 45th day

TABLE II.

| Patient No. | Body Wt. in lbs. | Age in years. | Basal requirements. | | Total quantity of food intake (g.) in 24 hours. | | | Total calorie intake* in 24 hours. (calculated). |
			Dreyer.	Beaumont & Dodds.	Carbo-hydrate.	Protein.	Fat.	
1	118	56	1334	1770	211.9	58.8	96.5	2007.3
2	126	42	1409	1890	505.9	106.2	43.7	2913.5
3	98	48	1217	1170	477.9	115.9	51.8	2915.2
4	120	48	1370	1800	375.6	110.7	84.9	2906.3
5	154	75	1518	2310	362.4	158.2	79.7	2877.2
7	112	32	1350	1680	477.3	103.7	61.8	2898.7
8	115	43	1400	1725	526.6	216.5	86.8	3976.9
9	113	50	1272	1575	436.8	135.8	73.7	3033.1
10	136	62	1115	2040	206.1	130.1	75.9	2083.3
11	125	49	1312	1875	273.1	144.2	89.6	2545.1
13	112	69	1255	1680	515.3	121.5	11.6	3131.1
14	120	50	1367	1800	317.0	83.9	28.2	1905.1
15	156	59	1515	2340	218.9	126.0	99.2	2336.7

*For calculation each g. of carbohydrate, protein and fat was taken equivalent to 4.1, 4.1 and 9.3 calories respectively.

Urine Analysis :

Morning sample of urine does not always give true indications of sugar percentage in case of a diabetic. Hence a sample for 24 hours was collected at regular intervals which would give total amount excreted in the whole day. From the percentage of sugar from such a sample we could very easily find out the total amount of reducing sugar eliminated from the system.

Estimation of Sugar and Albumin :

Sugar in urine was estimated according to the method of Benedict (23) as modified by Quick (24) and quantitative estimation of albumin, when present in the sample, was made according to the method of Esbach (Table IV).

Test for Acetone bodies :

Sometimes ketosis or formation of acetone bodies occur in the system of a diabetic (in condition of faulty fat metabolism) and their excretion in the urine is termed as ketonuria or acetonuria. Follin (25) points out that in strictly fresh urine, containing acetone bodies, the quantity of diacetic acid is 9-10 times that of acetone and the older the urine the greater becomes the relative proportion of acetone. Rothera's test is much more sensitive for diacetic acid than for acetone. The following modification of Rothera's test was used to give the most reliable test for acetone plus diacetic acid:—Morning samples of urine were used for these tests.

TABLE III—(Contd.)

Period of treatment in months.	Patient No. 11		Patient No. 12		Patient No. 13	
	U.S. %	B.S. mgm. %	U.S. %	B.S. mgm. %	U.S. %	B.S. mgm. %
0.0	0.00	121	7.69	345	7.13	349
0.25	0.20	...	6.25	335	6.00	...
0.5	0.00	120	3.57	326	3.8	226
1.0	0.00	113	4.7	315	1.6	218
1.5	...	...	3.1	293	...	...
2.0	0.00	108	...	...	1.31	205
2.5	...	...	...	...	...	...
3.0	0.00	85	...	...	0.8	206
3.5	...	...	...	...	...	...
4.0	0.00	85.2	...	...	0.3	218
4.5	...	...	...	...	...	...
5.0	...	...	...	...	...	...
5.5	...	...	...	...	...	...
6.0	...	...	...	...	0.00	217
6.5	...	...	...	...	...	...
7.0	...	...	...	...	0.00	165
7.5	...	...	...	...	...	...
8.0	...	...	...	...	0.00	161
8.5	...	...	...	...	...	...
9.0	...	...	...	...	0.00	160
9.5	...	...	...	...	...	...
10.0	...	...	...	...	0.00	155
10.5	...	...	...	...	...	...
11.0	...	...	...	...	0.00	116

TABLE III—(Contd.)

Period of treatment in months.	Patient No. 14		Patient No. 15	
	U.S. %	B.S. mgm. %	U.S. %	B.S. mgm. %
0.0	0.00	48.3	0.50	225
0.25	...	...	...	...
0.5	0.00	62.6	...	228
1.0	...	...	0.35	216
1.5	...	...	...	...
2.0	...	...	0.06	216
2.5	...	...	...	...
3.0	0.00	85	0.13	211
3.5	...	...	...	...
4.0	0.00	84	...	...
4.5	...	...	...	...
5.0	0.00	84.5	...	...

TABLE IV

Day of treatment.	Patient No. 1. Albuminuria mgms./100 cc.	Ketonuria. Roth.	Ketonuria. Ger.	Patient No. 5. Albuminuria mgms./100 cc.	Ketonuria. Roth.	Ketonuria. Ger.	Patient No. 6. Albuminuria mgms./100 cc.	Ketonuria. Roth.	Ketonuria. Ger.	Patient No. 8. Albuminuria mgms./100 cc.	Ketonuria. Roth.	Ketonuria. Ger.	Patient No. 12. Albuminuria mgms./100 cc.	Ketonuria. Roth.	Ketonuria. Gert.
0	Trace	0	0	10	0	0	20	+++	Pos.	True	++	V. Sl. Pos.	0	+++	Str. Pos.
1	...	...	...	...	...	...	...	++	Sl. Pos.	...	...	...	...	+++	Str. Pos.
3	...	...	...	40-	...	...	...	++	0	...	+	0	...	+++	Pos.
5	...	...	...	...	...	...	20	+	0	...	Trace	0	...	++	Pos.
7	...	...	...	...	...	...	...	0	0	...	Sl. Pos.	...	...	++	Sl. Pos.
9	Faint	...	...	...	...	...	...	Trace	0	Trace	...	0	...	++	V. Sl. Pos.
11	...	...	...	...	...	...	...	0	0	...	0	0	...	+	0
15	...	...	...	...	...	...	15	0	0	...	0	0	...	++	0
21	...	...	...	...	...	...	...	...	...	...	...	...	...	V. Sl. Pos.	0
28	...	...	...	...	...	...	...	...	...	...	...	0	...	0	0
35	Faint	...	...	...	...	...	...	...	...	...	...	...	...	+	0
42	...	...	...	...	...	...	...	...	...	...	...	...	...	V. Sl. Pos.	0
49	0	...	...	20	...	...	...	...	...	0	...	...	...	0	0
62	...	...	...	Trace	...	...	...	...	...	...	...	...	...	...	...
107	...	...	...	Faint 0	...	...	...	...	...	...	...	...	...	...	...
215 229	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

Carbohydrate Balance :

By the term carbohydrate balance is meant the difference between the total quantity of carbohydrate ingested along with food in a particular period of time (24 hours) and the sugar excreted in the urine during that period. Table V indicates the results with all the patients under investigation. It will be evident that the balance goes on increasing in all the cases thus indicating more and more utilisation of sugar in the system.

TABLE V

Patient.	Patient No. 1.				Patient No. 2.			
Day of treatment.	Initial.	80.	70.	211.	Initial.	30.	102.	230.
Total quantity of urine in 24 hrs. (litre)	3.5	1.7	1.5	1.2	2.2	1.6	1.4	1.2
Sugar p.c. of the urine (gms.)	5.7	1.2	0.01	0.00	2.95	0.43	1.66	0.012
24 hrs. glycosuria (gms.)	199.5	20.4	0.6	0.0	35.4	6.88	23.2	0.15
Total amount carbohydrate intake (gms.)	211.9	...	...	...	505.8	...	...	...
*Carbohydrate balance (gms.) (all "+")	12.4	109.5	211.3	211.9	469.9	498.4	182.1	505.1
Body wt. in lbs.	118	...	...	...	126	...	...	...

TABLE V—(*Contd.*)

Patient.	Patient No. 3.				Patient No. 4.			
Day of treatment.	Initial.	30.	70.	121.	Initial.	30.	71.	213.
Total quantity of urine in 24 hrs. (litre)	1.9	1.4	1.3	1.3	1.5	1.2	1.25	1.31
Sugar p.c. of the urine (gms.)	2.5	0.09	0.0	0.0	5.4	1.8	0.6	0.0
24 hrs. glycosuria (gms.)	47.5	1.26	0.0	0.0	81.0	21.6	7.5	0.00
Total amount carbohydrate intake (gms.)	477.0	...	...	...	375.6	...	...	...
*Carbohydrate balance (gms.) (all "+")	430.4	176.6	477.9	...	294.6	354.0	368.1	375.6
Body wt. in lbs.	98	...	...	...	120	...	...	...

TABLE V—(Contd.)

Patient.	Patient No. 5.				Patient No. 7.			
Day of treatment.	Initial.	30.	70.	230.	Initial.	32.	69.	101.
Total quantity of urine in 24 hrs. (litre)	2.65	2.7	2.5	2.2	2.45	1.83	1.8	1.5
Sugar p.c. of the urine (gms.)	1.9	1.9	1.02	0.8	2.5	2.5	1.0	0.3
24 hrs. glycosuria (gms.)	50.1	29.7	25.5	1.8	61.4	15.7	18	1.5
Total amount carbohydrate intake (gms.)	362.4	362.4	451.6	...	477.3	...	...	...
Carbohydrate balance (gms.) (all "+")	312.0	332.7	429.1	452.8	116.1	131.6	459.3	473.8
Body wt. in lbs.	151	...	...	...	112	...	...	...

TABLE V—(Contd.)

Patient.	Patient No. 8.			Patient No. 9.			Patient No. 10.		
Day of treatment.	Initial.	30.	50.	Initial.	30.	59.	Initial.	30.	84.
Total quantity of urine in 24 hrs. (litre)	3.2	2.4	2.0	2.58	1.6	1.3	2.4	1.8	1.5
Sugar p.c. of the urine (gms.)	4.35	2.5	2.5	2.5	0.4	0.07	3.3	0.9	0.12
24 hrs. glycosuria (gms.)	139.2	60.0	50.0	64.5	6.4	0.91	79.2	16.2	1.85
Total amount carbohydrate intake (gms.)	526.6	...	...	136.8	...	...	206.1	...	...
Carbohydrate balance (gms.) (all "+")	387.4	466.6	476.6	372.3	430.4	435.9	133.9	189.9	204.3
Body wt. in lbs.	115	...	...	105	...	...	136	...	...

TABLE V—(Contd.)

Patient.	Patient No. 11.		Patient No. 13.			Patient No. 12.		
Day of treatment.	Initial.	30.	Initial.	11.	38.	Initial.	16.	66.
Total quantity of urine in 24 hrs. (litre)	1.6	1.3	3.18	2.8	2.4	4.00	2.2	1.3
Sugar p.c. of the urine (gms.)	0.90	0.00	6.25	6.25	4.2	7.13	3.8	1.2
24 hrs. glycosuria (gms.)	14.4	0.00	198.8	175	100.8	285.2	83.6	15.6
Total amount carbohydrate intake (gms.)	273.1	...	...	...	...	543.3	...	...
*Carbohydrate balance (gms.) (all "+")	258.7	273.1	...	...	...	260.1	461.7	529.7
Body wt. in lbs.	125	...	...	...	...	112	...	...

TABLE V—(Contd.)

Patient.	Patient No. 14.		Patient No. 15.	
Day of treatment.	Initial.	88.	Initial.	20.
Total quantity of urine in 24 hrs. (litre)	1.5	1.4	1.3	1.2
Sugar p.c. of the urine (gms.)	0.0	0.0	1.8	0.82
24 hrs. glycosuria (gms.)	0.0	0.0	23.4	9.84
Total amount carbo-hydrate intake (gms.)	317	...	218.9	...
*Carbohydrate balance (gms.) (all "+")	317	...	195.5	209.1
Body wt. in lbs.	120	...	156	...

(Calculated according to Joslin, without taking protein and fat into consideration).

Statement of Patients :

Apart from the clinical results obtained from the analysis of urine and blood, occasional statements of the patients were also collected in presence of recognised and experienced medical officers, in some specific forms, and these were signed by the patients and countersigned by the said medical officers. Table VI shows a synopsis of some such statements. This is to be noted that two out of these fifteen patients (P_5 & P_{13}) who are old men over 70 years of age at present and had been suffering for a long period, lost all hopes of their lives even in spite of taking insulin regularly and were almost at the points of death, have also come to the lives of activity through ingestion of amellin. P_5 was bed-ridden for about one year and he is now taking morning walk alone. P_{13} became unconscious almost every day and had 7.1% sugar in urine. He is also in a perfect state of health at present. P_2 who had a boil operated and was insisted upon taking recourse to insulin by local surgeons, succeeded in having his wound healed up through amellin alone within a very short period.

TABLE VI

Patient No. profession.	Age (yrs.)	Duration	Statement on	General nervous condition.	Teeth.	Eyes.	Appetite and bowels.	Frequency of urination.	Sleep.	Whether dizziness of the brain.	Joint pain etc.	General remarks.
1	2		3	4	5	6	7	8	9	10	11	12
1. Medical Officer.	56	6	Initially on 29.7.41.	Extreme weakness.	...	...	...	Polyuria.	Not sound.	Yes.	...	
			Finally on 4.9.41.	Much improved.	...	...	...	Normal.	Practically sound.	No.	...	General look improved much and feeling stronger day by day.
2. Business man.	42	3	5.8.41.	Very weak, postrate.	...	...	Constipative.	Polyuria.	Not sound.	Yes.	...	...
			3.12.41.	Much improved.	...	...	Practically normal.	Almost normal.	Sound.	Only on rare occasion.	...	Greatly improved.
3. A Hindu widow.	48	1	6.9.41.	Extreme weakness.	Occasional pain.	...	Not much.	Polyuria.	Not at all sound.	Yes.	Occasional pain on the limbs.	Anæmic.
			7.3.42.	Much better.	No troubles.	Sight greatly improved.	Increased.	Normal.	Sound.	Condition greatly improved. Yes.	Abated.	Almost normal.
4. School Master.	46		15.9.41.	Extreme weakness.	Serious symptoms of pyorrhœa.	Rapid fall of eye sight. Could not recognise persons from about 10 ft.	Very small.	About 5 to 6 times.	Unsound.	Yes.	...	...
4. School Master.	46		30.9.41.	Now I feel I have almost no attack of pyorrhœa.	...	...	...	...	...	...	...	...
			22.12.41.	Sight greatly improved, former condition restored.	...	...	...	...	...	...	...	...
			3.2.42.	Feeling better day by day.	No trouble.	Normal condition restored.	Normal.	Normal.	Sound.	No.	...	...

TABLE VI—(Contd.)

Patient No. profession.	Age	Duration (yrs.)	Statement on	General nervous condition.	Teeth.	Eyes.	Appetite and bowels.	Frequency of urination.	Sleep.	Whether dizziness of the brain.	Joint pain etc.	General remarks.
1	2	3		4	5	6	7	8	9	10	11	12
5. Retd. Govt. Officer.	75	25	4.12.11.	Extreme weakness. Can't rise from bed.	...	Long sight good, can't read books without spectables.	...	21 times (polyurea).	Not sound.	Yes, accompanied with constant headache.	...	Pus and blood excreted from the wound of the piles.
			5.2.12	Now in a position to rise from bed and can walk alone.	...	A bit improved.	...	10 time in 24 hrs.	Improved.	No headache now.	...	Feeling of general improvement; condition of the wound better.
7. Money Lender.	33	5	9.1.12.	Feeling very weak and depressed.	Swelling of the gum every now and then. No pain now.	Defective vision specially at night.	...	Polyuria.	Not sound.	Yes.	Feeling occasionally.	...
			28.2.12.	Much improved.	...	Better.	...	Number of times and quantity decreased.	Without opium good.	No pain now.	Diminished. Look improved.	
8. Clerk in Govt. Office.	43	9	28.2.12.	Feeling very weak.	...	...	...	9 times a day.	Not sound.		Joint pain present Acetone bodies —present.	A sore on the leg. suffering for about a month.
			7.3.42.	Feeling a little better.	...	...	...	7 times a day.	A bit sound. Condition better.		Joint pain improved. Acetone bodies—absent.	Sore completely healed up.
13. Retd. Officer.	69	10	18.3.12.	Very weak.	...	...	Constipative, moved after 2 or 3 days by taking purgative etc.	...	...	Yes.	Occasionally.	...
			7.3.42.	Feeling better.	...	...	Moving regularly without purgatives.	...	...	No dizziness.	Pains in the fingers abated.	General look far better.

Figures 1-10, showing graphically relative lowering of hyperglycemia and glycosuria.
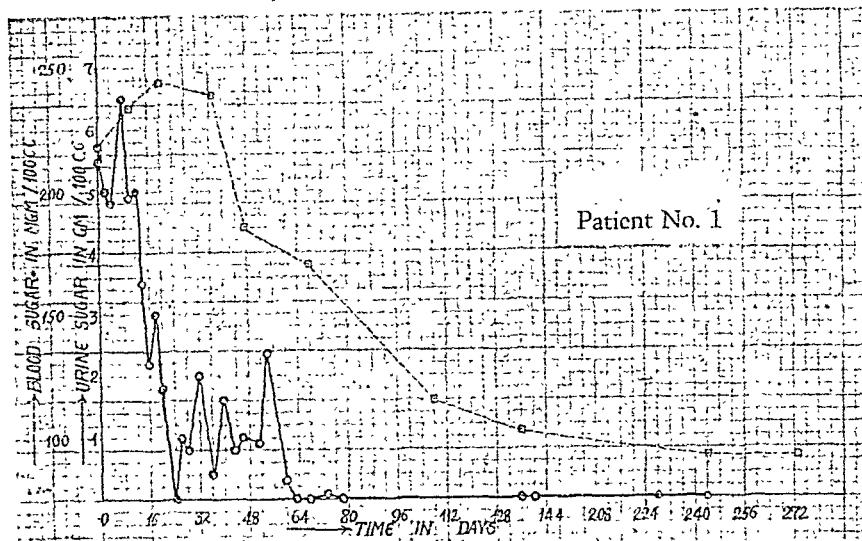
Fig. 1
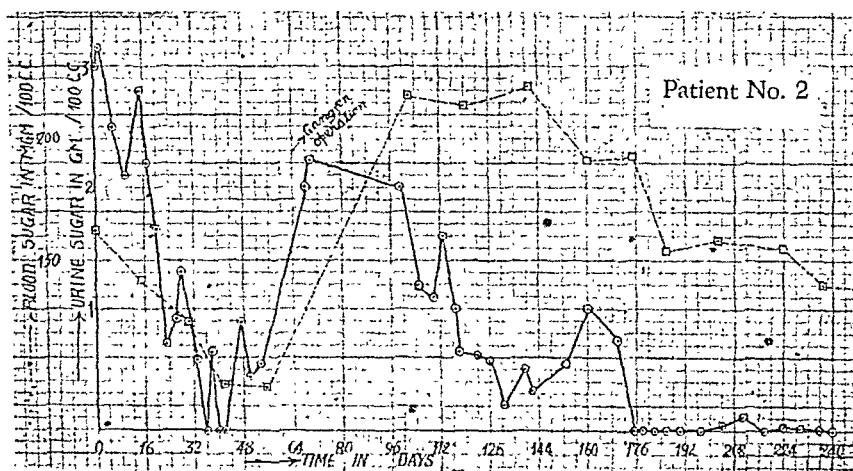
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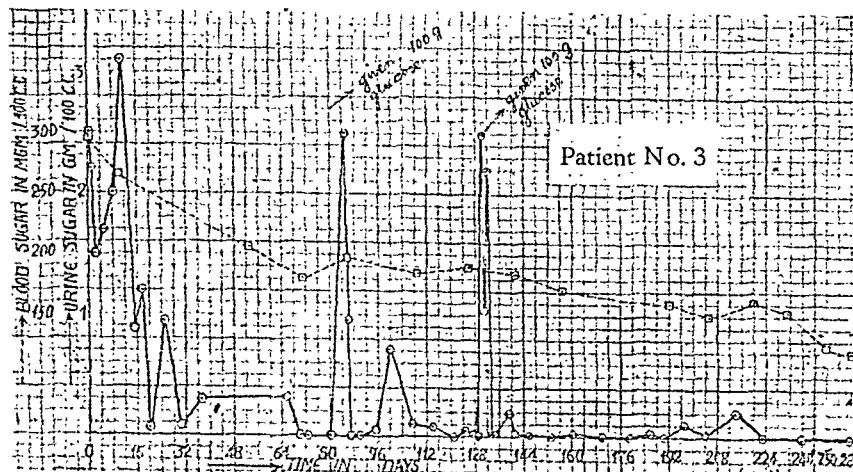
Fig. 8
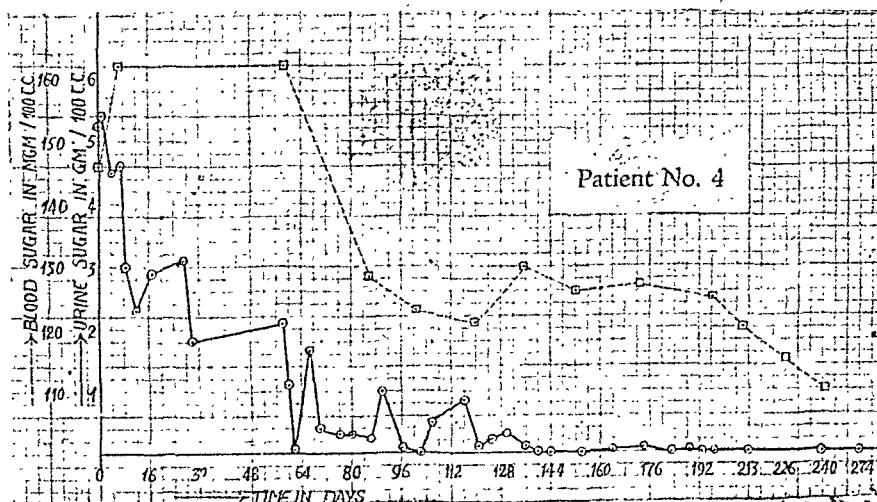
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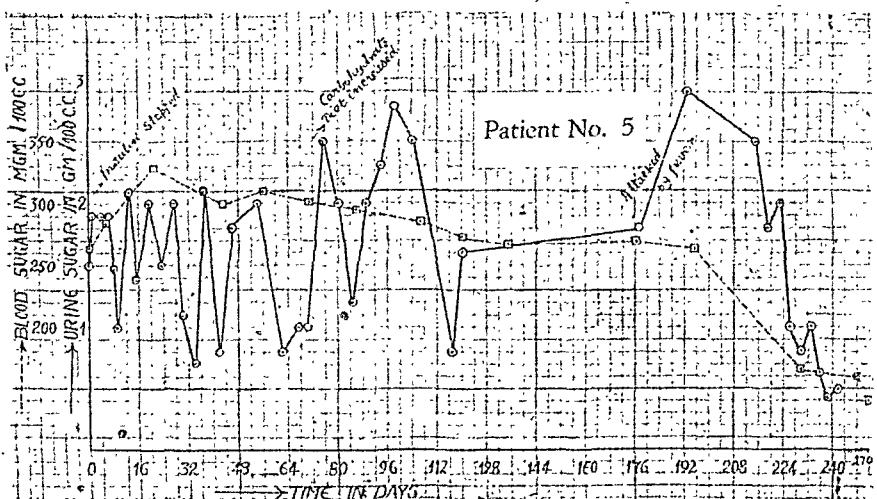
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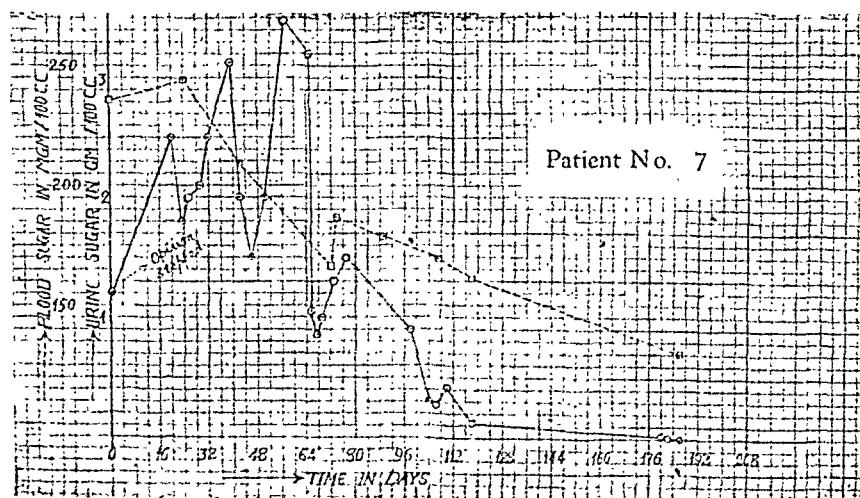
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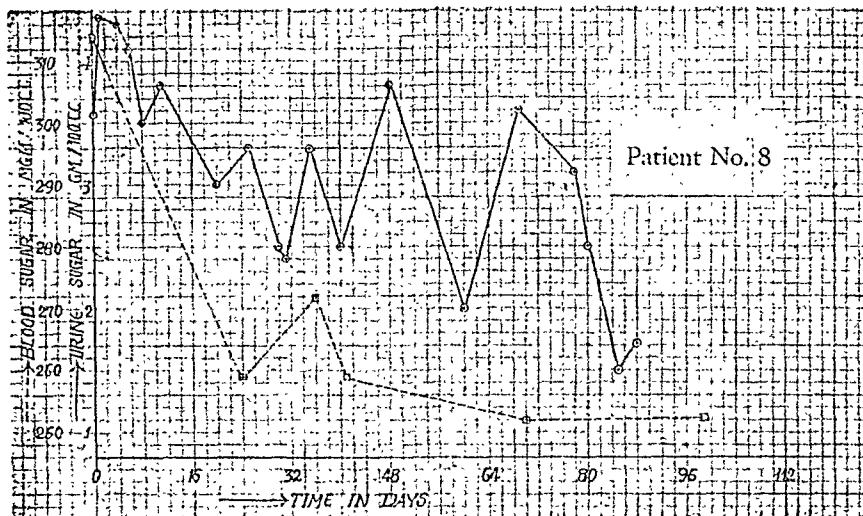
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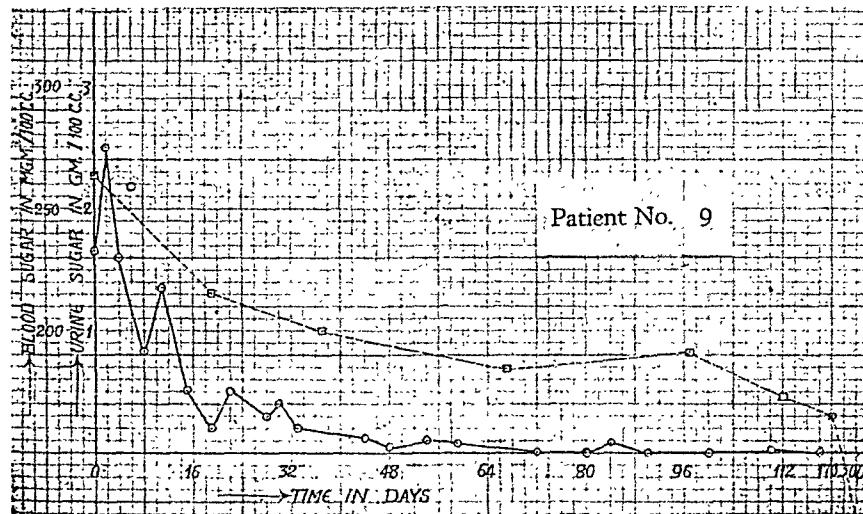
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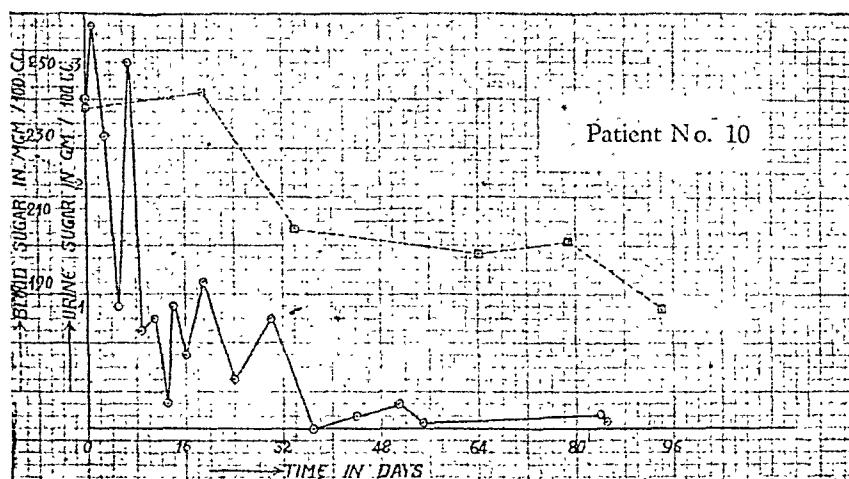
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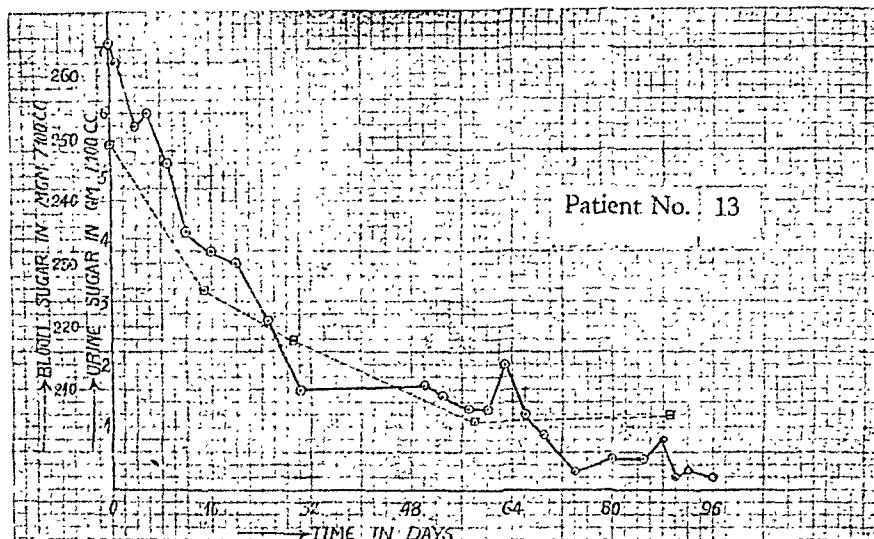
Fig. 10

DISCUSSION OF RESULTS

(1)   *Glycosuria and hyperglycemia :*

It is evident from the tables that even inspite of allowing enormous quantity of carbohydrate diet to the diabetes patients, there has always been a steady and gradual fall in glycosuria and the beneficial effect has been observed within a period of fortnight or so.   In case of P_{13} the initial value of sugar per 100 cc. urine was 7.13 (total quantity in 24 hours being 4000 cc.).   But to our utter surprise the value came to 1.6% (quantity in 24 hours—1500 cc.) withon one month's time.   Results with P_1 and P_4 who had 5.7% (3.5 litres) and 5.4% (1.5 litres) sugar in urine at the beginning, were found to be highly satisfactory within one month's time only, when these values came down to 1.20% (1.7 litres) and 1.8% (1.2 litres) respectively (Table III).   There is not only a fall in percentage of sugar in urine but there is also a rapid fall in the total amount of urine in 24 hrs., thus bringing the daily glucose excretion to a very small value, which indicates enhanced utilisation of glucose in the system.   Table V will show the results of carbohydrate balance.

Amellin differs in its action, with insulin regarding the fall of blood sugar. Insulin, which is effective by injection alone, may sometimes lead to such hypo- glycemic condition, just within a short period of injection, that it may be necessary, in order to avoid convulsion, to administer glucose orally.   The sugar in urine also vanishes consequently but the result is temporary and one has to go on, taking this, throughout the whole course of one's life time.   While amellin (by oral administration) first exerts its influence in relieving the symptoms of glycosuria at the outset slowly and causes steady fall in hyper-glycemia sometimes after a few weeks have passed and the blood sugar value under no condition falls below the normal limit.   This slow action may be due to two reasons:—

(a)   The patients lived in their respective houses and took their usual diet thus taking a carbohydrate diet far exceeding the limit recommended

by authorities during diabetes. Joslin (21) states "The total carbohydrate in the diet of a diabetic patient under treatment varies with the physician, but I believe it should seldom be less than 100 gms. or more than 200 gms." But no patient of ours had an intake of carbohydrate less than 211 gm. in 24 hrs. ; the highest amount being 545 gms. (Table II).

(b) Amellin might have something to do in neutralising or doing away with toxic substances generated within the system, which would require some time. Further works, to elucidate this point, are in progress.

(2) *Furunculosis, sores, gangrene etc. :*

Many a time a carbuncle or during a crop of boils, suspicion to the diabetic condition of a person, necessitates examination of urine and blood for sugar etc., for proper diagnosis of diabetes. It has long been established that condition of hyperglycemia lowers the resisting power of an individual and makes him liable to pyogenic infection. It has been observed that sometimes a mere scratch may lead to cellulitis or even erysipelas.

It is believed that increased concentration of sugar in the tissues acts as a culture medium for the staphylococcus. We know from the works of Follin, Trimble and Newman (27) that a part of the glucose injected into an animal is stored in the skin. The patient No. P_2, who had an attack of a severe type of boil on the 73rd day, recovered soon only through the effect of amellin and the wound after operation completely healed up within three weeks. Another wound of Patient No. P_8 who had been suffering from that for about a month or so even in spite of applying ointments etc., healed up completely within 6 days of coming under our medication. The wound of P_3 also healed up completely on the 8th day. P_5 had old piles which excreted pus and blood regularly but the condition of the wound became better only in a few days time. P_6 had been suffering from a gangrene in the left toe for the past two months, condition of which was improved greatly within 15 days time and the wound completely healed up within next 15 days. Thus amellin by some way or other, has the capacity of increasing body resistance to a very great extent.

(3) *Pyorrhœa :*

It is really interesting to note that amellin is also highly beneficial in removing pyorrhœa accompanied with diabetes. The statement of a Patient No. P_4 after only a fortnight "Now I feel I have almost no attack of pyorrhœa" confirms this point satisfactorily. P_7, who had swelling of the gum every now and then, recovered completely, within a very short time (Table VI).

(4) *Eye troubles* (retinitis etc.) :

Several types of eye troubles may be associated with diabetes. Rudy and Sachs (28) and Fishcher (29) discussed the transitory visual disturbance in diabetes. Braun (30) investigated 700 diabetes of whom 111 showed changes in retina in the form of retinitis diabetica. We too observed this trouble with our patient P_4, who could not recognise persons from a distance of about 10 ft., when he first

came to us. To our utter surprise we learnt from his statement that his eye-sight became greatly improved within a fortnight's time and his normal vision was restored after three months' treatment. Improvements in the eye eight were also observed in case of patients P_3 and P_7 (Table VI).

(5) *Neuritis :*

Detailed studies of Jordan (31) shows the frequency of diabetic neuritis in patients. In case of P_3 and P_7 the joint pain involved the legs only while in P_{13} generalized involvement of legs and fingers caused severe pain to occur, especially during night time. Relief was observed in all cases after a few weeks treatment with amellin.

(6) *Obesity :*

When there is disturbance in the metabolism of carbohydrates, as is the case in diabetes, greater difficulty is experienced by the system in oxidising fat, which when taken in excess has a general tendency to be stored in the different fat godowns of the body. Again if a diabetic goes on taking too much carbohydrate the hyperglycemic condition goes on increasing at the outset, and ultimately there is developed mechanism of conversion of sugar into fat which, for failure of being oxidised, gets accumulated in different parts of the body thus giving an ugly appearance of obesity. The localities of fat deposition are:—Omentum, serous membranes, space between the skin and muscles, muscles and finally the organs especially the liver and the heart.

Both the female patients P_3 and P_9, who are hindu widows and generally accustomed to high carbohydrate and low protein diets, suffered much through obesity. It was very difficult for them even to walk a few yds. without feeling exhausted. They were highly anæmic too. Reduction of body fat has been observed in both these cases. They are now almost in normal health and vigour. P_3 a patient of 75 yrs. of age (at present) who was bed-ridden and was pot-bellied is now in a position to get down to the ground floor alone and can walk freely without the help of others. These prove beyond doubt that amellin not only helps proper utilisation of food carbohydrate but can also bring about oxidation of stored body fat which is a curse to the rich civilised individual.

(7) *Heart Troubles :*

P_9, who was suffering much from heart troubles associated with diabetes, has recovered satisfactorily. According to the physicians there was a deposition of fat on all sides of her heart. She is now free from all heart troubles and anxieties.

(8) · *Ketonuria :*

There was excretion of acetone bodies (Ketonuria) in the urine of some severe cases (Table IV). Dakin (32) holds that acetoacetic or diacetic acid is an intermediate product of faulty oxidation of fatty acids. It is believed that under normal conditions of metabolism the end products are carbon dioxide and water.

It was observed that in cases of P_6 and P_8 urine became free from acetone bodies within a fortnight, and in case of P_{12}, where Rothera's test was initially

found to be "+ + + +", ketonuria was absent on the 21st day. Thus relief in obesity as well as in ketonuria indicates definitely the high oxidative value of amellin in the human system. Further work on the reduction of body fat in the experimental animals and change in its compositions etc. are in progress.

(9) *Acidosis :*

Acidosis which has broadly been defined by Peters and Van Slyke as an abnormal condition caused by the accumulation in the body of an excess of acid or the loss from the body of alkali, is one of the symptoms associated with diabetes. It is generally caused in a diabetic, through the gradual accumulation of β-hydroxy butyric acid, aceto-acetic acid and crotonic acid which are the intermediate products of faulty oxidation of fatty acids and loss of alkali reserve for their neutralisation etc. As this symptom is closely associated with ketonuria, patients when relieved of the latter, got rid of the former too.

(10) *Albuminuria :*

Joslin (22) observed that amongst the diabetic suffering for a long period of 20 years, there was noticeable amount of albumin in about 26% cases, while in cases of less than two years' duration only 4% patients had the symptoms of albuminuria.

Among our patients, P_1, P_3 and P_6 had detectable amounts of albumin in their urine. P, who had been suffering for the last 25 years from diabetes, and had the highest value *i.e.* 40 mgs. per 100 cc., required about 4 months' treatment with amellin to get rid of albuminuria, while the other patients required only a few weeks' time (Table IV).

SUMMARY

(1) Effect of amellin in reducing glycosuria and hyperglycemia on fifteen more diabetic patients, has been recorded.

(2) Unlike insulin it has been found to cause a slow but steady fall in blood sugar, time required to reach the normal limit varying according to duration and virulence of the disease. Urine sugar also falls gradually till it disappears.

(3) Amellin can also bring about relief in acetonuria, albuminuria, acidosis and other complicated troubles associated with diabetes.

(4) It can exert its physiological influence, even in the daily dose of 15 to 20 mgms., on patients taking no heed to strict regulation of diets. Some patients were taking more than 3,000 calories a day, yet they showed marked improvements and signs of recovery.

(5) Property of amellin in healing wounds (*e.g.* sores, gangrene, boils etc.) has called for its special significance in regeneration of body tissues.

(6) That amellin can bring about proper fat metabolism, has also been indicated from reduction of fat from the adipose tissues.

(7) Amellin seems to bring far more permanent relief in diabetes than insulin or any of its preparation.

ACKNOWLEDGEMENT

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NICOTINIC ACID CONTENT OF BLOOD IN DISEASES. PART II. PELLAGRA.

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Pellagra is not very prevalent amongst the wheat-eating populace of the Punjab. Only 5 cases in the course of three years could be obtained for investigation. All the same it is by no means a rare disease. There were 6,333 and 5,853 reported deaths from pellagra in the United State of America in 1930 and 1931 respectively, but it is not inconceivable that the actual number of deaths from pellagra might be considerably higher. Pellagra has been known to exist in Italy, Rumania, Russia, Spain, Portugal, France, Egypt and many other countries.

Pellagra is a nutritional deficiency disease associating itself principally with the subnutrition of nicotinic acid itself or its derivatives. It is mainly encountered in endemic and sporadic forms in poverty-stricken communities and in chronic alcoholics respectively. It also occurs among individuals with antecedent organic disease, which in its turn interferes with the ingestion, assimilation or utilization of a well balanced and adequate diet. This deficiency of nicotinic acid involves a great derangement of the fundamental processes of the cells. The activities of the body cells depend upon the energy changes in the oxidation and reduction reactions in which the metabolite becomes activated on collision with a specific protein containing nicotinic acid amide-triphospho pyridine nucleotide. Thus diseases like pellagra resulting from nicotinic acid deficiency may retard the energy changes in these cells with consequent decrease of the cell activities.

Variations of nicotinic acid content of the various constituents of blood, which is the main source of supply of nicotinic acid to tissues, have been investigated in patients suffering from pellagra and attempts have been made to correlate laboratory findings with other clinical symptoms.

RESULTS AND DISCUSSION

The methods followed for estimating nicotinic acid in blood and urine were as described by Kochhar (1) and the results relating to the five cases are summarised in Table I.

B. D. KOCHHAR

TABLE I
Nicotinic acid content of blood in pellagra.

Dates.	Packed cell volume. %	Nicotinic acid.			
		Blood $\mu g\%$	Plasma $\mu g\%$	Cell.**	Urine 24 hours mg.
Case No. 1.					
11th* March 1910	...	384	...	...	...
12th* ,,	...	315	...	...	...
13th* ,,	44	320	...	...	...
15th ,,	...	380	...	...	...
16th ,,	...	300	...	...	...
18th ,,	...	400	...	...	...
4th April	44	412	...	...	...
Case No. 2					
23rd* January 1942	32	184	20	534	0.69
24th* ,,	32.2	168	traces.	521	traces.
25th ,,	...	320	20	...	...
26th ,.	...	450	...	...	...
27th ,,	34	220	22	610	0.72
28th ,,	34	256	50	656	...
29th ,,	...	344	183	657	...
30th ,,	...	...	...	...	3.1
31st ,,	36	320	120	670	2.1
1st February	...	...	...	...	4.9
2nd ,,	...	152	66	...	1.2
3rd ,,	...	...	...	...	2.1
4th ,.	...	...	...	...	1.2
5th ,.	37	264	115	518	2.1
6th ,,	...	...	...	...	0.8
8th ,,	...	...	...	...	2.2
9th ,,	...	...	...	...	1.5
10th ,,	...	...	...	...	1.2
11th ,,	37	532	105	448	1.4
Case No. 3.					
25th* February	28	280	65	833	...
26th* ,,	31	218	16	780	0.3
27th* ,,	...	...	...	...	0.5
28th ,,	31.8	256	10	729	4.3
1st March	...	...	...	...	18.2
3rd ,,	...	...	...	...	8.5
4th ,,	30.5	560	150	1194	9.5
5th ,,	...	...	...	...	16.7
6th ,,	...	...	...	...	9.4
7th ,.	...	...	...	...	7.0
8th ,,	...	...	...	...	19.8
9th ,.	32	432	80	1180	5.8

TABLE I—(Contd.)

Dates.	Packed cell volume. %	Blood μg%	Plasma μg%	Cells.	Urine 24 hours mg.
		Case No. 4			
21st April 1942	31.1	317†	Nil	1014‡	1.0
		Case No. 5.			
20th* May	28.5	300	120	751	...
23rd ,,	28.0	280	110	717	traces.
24th ,,	...	290	...	...	0.6
26th ,,	...	...	...	...	3.9
27th ,,	...	...	...	...	9.7
28th ,,	34.8	440	200	600	
					blood was taken 15 mins. after the test dose.
30th ,,	...	...	...	...	5.4
2nd June	...	...	...	...	4.0
3rd ,,	...	...	...	...	3.0
		Case No. 6.			
30th December	...	...	...	...	Nil.
3rd January 1943	37	...	...	...	traces.
4th ,,	...	270	60	630	traces.
5th ,,	...	250	95	515	1.0 mg.
9th ,,	...	...	...	...	traces.
10th ,,	...	...	...	...	0.7
11th ,,	...	...	...	...	3.3
13th ,,	40	400	110	835	7.0
14th ,,	...	...	...	...	5.3
15th ,,	...	...	...	...	4.8
16th ,,	...	...	...	...	2.6
22nd ,,	...	460	...	...	...
23rd ,,	40	490	150	1000	11.3
29th ,,	40	500	140	1040	13 in 6 hours.

*Before the start of nicotinic acid therapy.
**Calculated as 100 (*Blood value-plasma value*)+plasma value packed cell volume.
†Calculated from cell nicotinic acid.
‡Experimental.

5

Blood :—The average nicotinic acid content of whole blood in 6 cases of pellagra (Table I) was 277μg per cent. ranging from 184 to 384μg percent. This average is lower than the average in normal or anæmia cases, which was 417μg per cent. ranging from 240 to 850μg per cent., and 326μg per cent. ranging from 199 to 480μg per cent. respectively, Kochhar (2). But it fell except for case No. 2, in the lower range of the distribution of nicotinic acid in blood in normal or anæmia cases. The average nicotinic acid content of plasma in pellagra was found to be 39μg per cent. which is low as compared with the average in anæmia (131μg per cent.) and normal subject (68μg per cent.). The average concentration of nicotinic acid in blood cells in pellagra was 737μg per 100 mil. of cells—a figure slightly lower than the average value for normal subjects (850μg per cent.), but fairly low when compared with the average value for anæmia cases (1426μg per cent.).

Since it has been thought Kochhar (2) that the ratio between the nicotinic acid concentration in blood cells and in plasma tends to remain constant, it is probable that low plasma values in pellagra are due to low concentration of nicotinic acid in cells. In cases Nos. 2 and 3 Table I, as also in other cases, the plasma value increased gradually after the nicotinic acid therapy, though— in the former the concentration of nicotinic acid decreased from 565 to 449μg per cent. and in the latter it increased from 780 to 1494μg per cent. However, in both these cases there was an increase in the hæmatocrit value (30 to 37 and 28 to 32 respectively). Case No. 3 responded markedly to nicotinic acid therapy. Brief protocols of these two cases are given in the end of this paper.

Though the nicotinic acid content of blood in pellagra is very low yet in the majority of cases it falls within the lower limit of the range in normal subjects, and a continuous ingestion of nicotinic acid has always produced a slight gradual increase in the nicotinic acid content of blood, no matter whether the initial value was high or low or the subject was normal or a pellagrin. Thus the level of nicotinic acid in the blood is not necessarily the best indicator of pathological processes in the tissue for nicotinic acid deficiency.

Graphs 2-A and 2-B show the post absorptive concentration of nicotinic acid in the blood of normal subjects and pellagra cases ; A relates to normal persons when the daily intake was 200 mg. of nicotinic acid per day per dose and B to pellagra cases when 400 mg. of nicotinic acid in 4 doses of 100 mg. each was given daily. An examination of graph 2 shows that nicotinic acid of blood increased with test doses in all cases but the rise was more conspicuous in normal subjects than in pellagrins ; this difference is particularly marked after the first one or two doses. It took 3 to 4 days or even less in normal subjects and 5 to 6 days or even more in pellagra cases before a saturation stage—when the rise was very inappreciable, if at all, was reached. It has not, however, been tried how much daily minimum intake of nicotinic acid by normal subjects would be required to keep up its saturation level which may be 400 μg per cent. at the minimum as seen from the graphs.

Urine :—In normal individual with moderate to liberal amounts of nicotinic acid in diet (30 to 100 mg.) the amount of urinary excretion of nicotinic acid,

A. NORMAL
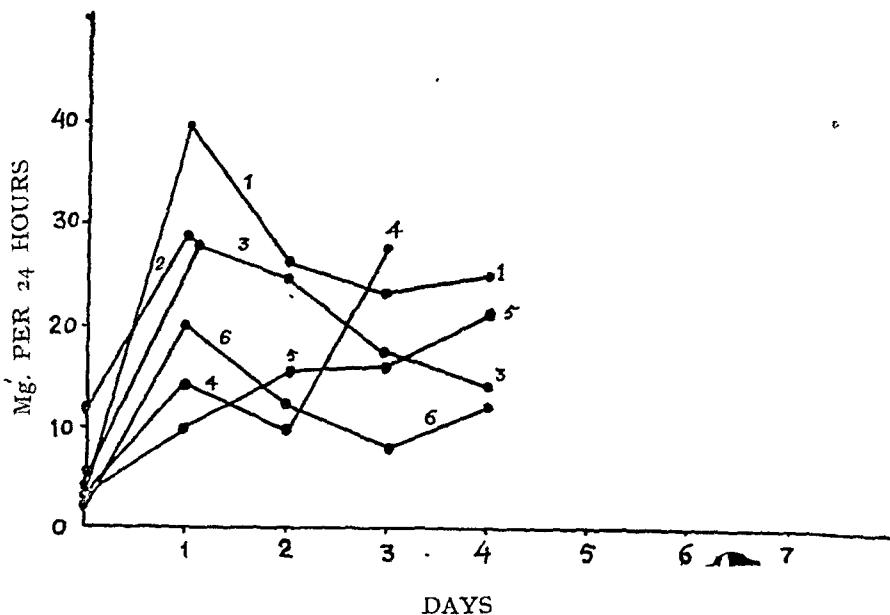
B. PELLAGRA
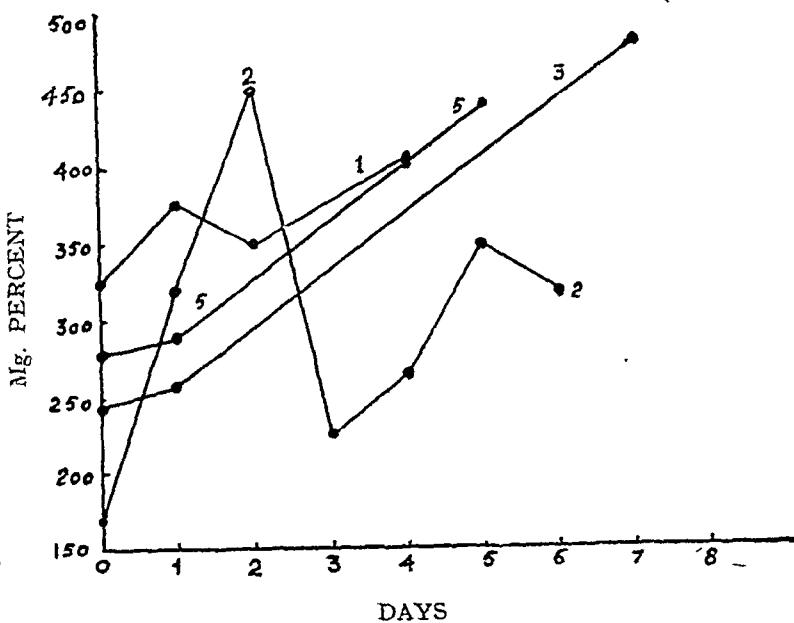
GRAPH. 2.
NICOTINIC ACID CONTENT OF BLOOD AFTER A
CONTINUOUS INTAKE OF TEST DOSES.

A. NORMAL
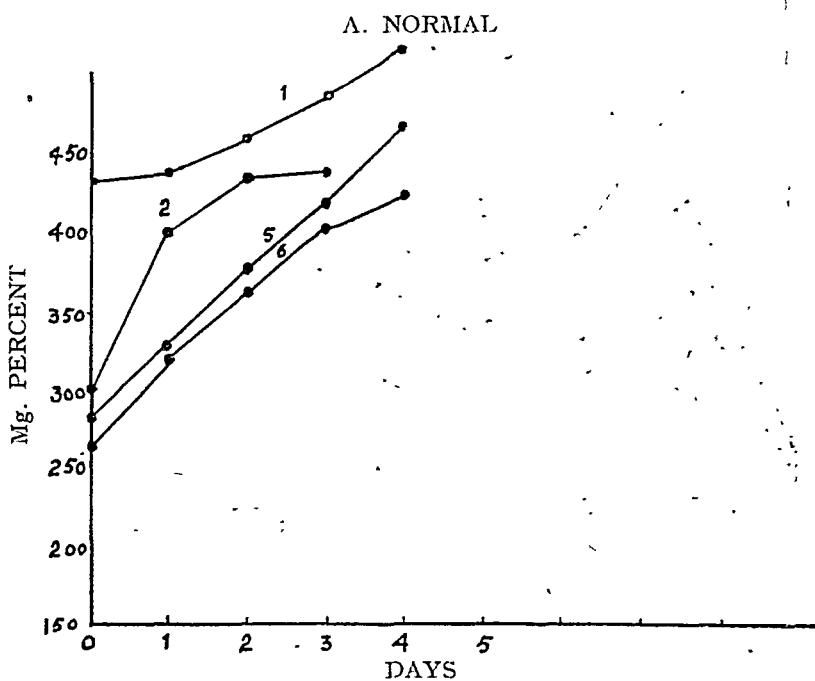
GRAPH. 3
NICOTINIC ACID EXCRETION IN URINE

B. PELLAGRA
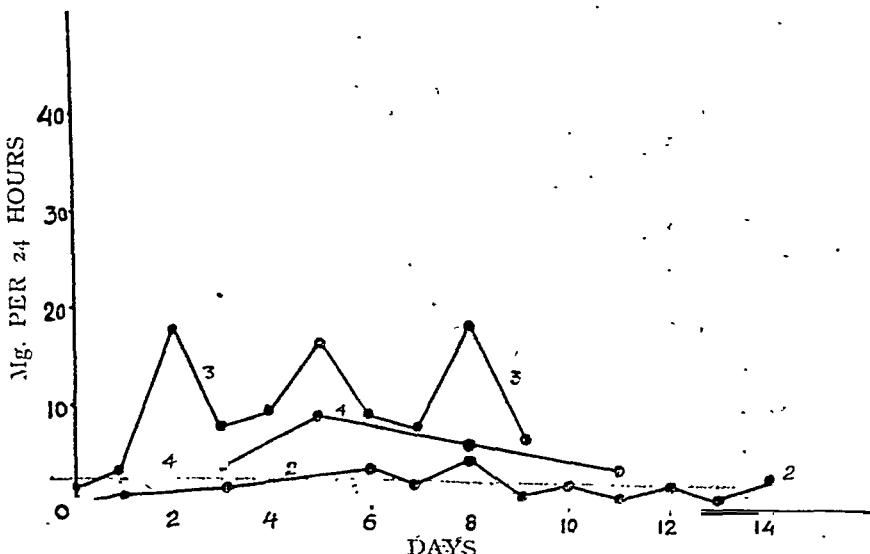
ranges from 3 to 11 mg. per 24 hours, Kochhar (3). On the other hand when the diet is low in nicotinic acid or the absorption is defective, the daily urinary output falls below 3 mg. In pellagrins only a trace of nicotinic acid in urine was observed but after the intake of nicotinic acid for several days continuously the output of nicotinic acid in urine rose to normal in most of the cases. Field *et al* (4) have shown a marked and a constant decrease in the urinary excretion of trigonelline in deficient subjects whereas the excretion of free nicotinic acid or its amide in urine remains practically constant in normal subjects or pellagra cases. The urinary excretion of trigonelline according to these authors, may be a measure of the nicotinic acid nutrition. The method followed in this investigation for estimating nicotinic acid in urine measures the total excretion of the nicotinic acid group which includes free nicotinic acid, nicotinic acid amide, nicotinic acid and trigonelline.

However, mere determination of the daily excretion of nicotinic acid is of limited value as a help in the investigation of nicotinic acid deficiency. Better information could be obtained by the study of the effect of a standard dose of nicotinic acid on the daily output. The changes in the amount of nicotinic acid excreted in the urine of normal and deficient subjects before and after the nicotinic acid therapy may, therefore, reflect the state of saturation of the tissues. It requires a week or more on a high nicotinic acid intake before the response to a test dose becomes marked. This is illustrated in Table II.

TABLE II

Excretion of nicotinic acid in urine before and after intake of "test doses"

Serial Nos.	Nicotinic acid excretion in 24 hours.		
	Initial mg.	After one 'test dose' mg.	After several 'test doses' mg.
(a) *Normal subjects.*			
1	7.0	40.6	21.2
2	10.8	28.9	...
3	5.4	28.7	14.1
4	4.5	14.9	27.8
5	4.8	11.5	22.5
6	3.0	21.9	13.8
Average	5.9	24.4	20.1

(b) *Pellagra cases.***

Serial Nos.	Initial mg.	After one 'test dose' mg.	After several 'test doses' mg.
1	0.69	0.72	3.0
2	0.80	4.3	19.8
3	traces	0.6	9.7
4	traces	traces	13.0
Average	0.25	1.4	11.4

*'Test dose' was 200 mg. per day per dose.
**'Test dose' was 400 mg. per day in four doses of 100 mg. each.

Graphs 3-A and 3-B show the changes in the excretion of nicotinic acid in urine after 'test doses' of 200 mg. per day per dose in normal subjects and 400 mg. per day in 4 doses of 100 mg. each in pellagra cases. In normal subjects the increase in the output of nicotinic acid in urine after a 'test dose' is very marked, while in pellagra cases it is very insignificant.

Unlike blood, excretion of nicotinic acid in urine seems to be a measure of the nicotinic acid deficiency in the tissues. In pellagrins or in nicotinic acid deficient cases the daily excretion of nicotinic acid in urine falls to a very low value or even to traces, which does not improve with a single test dose. But with a continuous intake of 'test doses' there occurs a gradual increase in its output. On the other hand, in subjects having no nicotinic acid deficiency the output of nicotinic acid in urine is conspicuous even after a single dose. Therefore, whereas the estimation of nicotinic acid content of blood is probably of little value in the diagnosis of nicotinic acid deficiency, its estimation in urine may help better in the understanding of the state of this vitamin in the body.

Observations have been made previously Kochhar (3) that most of the nicotinic acid after its absorption is removed from the blood. Part of it (10 to 20%) is excreted by the kidney and the rest is either retained or used up in the body. Excretion of nicotinic acid in urine in pellagrins after a test dose is insignificant (Table I). Thus body retains more nicotinic acid in pellagra than normal subjects. Perhaps in pellagra the nicotinic acid is retained by organs such as liver, muscles which have been shown by Kohn et al (5 and 6) and Axelrod et al (7, 8 and 9) to the depleted in experimental black-tongue and in human pellagra. The increased concentration of coenzymes containing nicotinic acid amide in these organs in canine black-tongue after ingestion of nicotinic acid has already been demonstrated by Axelrod et al (7-9).

Following are the brief protocols of the cases Nos. 2 and 3 (Table II):—

Case No. 2-B.A. male, aged 25, admitted on January 19, 1942, complaining of frequency of stools (duration 6 months) ; dryness and tightness of skin (duration

4 months) and papular eruption on trunk, arms and legs (duration 20 days). He was a labourer living on poor diet consisting of wheat and maize bread with occasional meat and vegetables. About six months before admission the patient had fever with shivering ; it was intermittent and would subside by sweating ; along with it he began to have about ten loose motions a day which contained blood and mucus and were accompanied by griping and severe straining. Fever and dysentery subsided after about a fortnight, but both appeared off and on at irregular intervals. For 20 days before admission to the hospital he had been having 3 or 4 stools a day without any blood or mucus. He had had no fever for two and a half months. About four months back he also began to observe dryness, tightness and pigmentation of skin of the exposed parts, i.e., dorsum of the hands, feet, face and neck ; gradually scales began to fall off from these parts of the body. 20 days back he got papular eruption on trunk, arms and legs. There was no history of itching. The hæmoglobin content, hæmatocrit value, red blood cell count, white blood cell count were 50%, 32%, 27,90,000 per c.mm. and 4,800 per c.mm. respectively. His liver and spleen both were enlarged. He was given nicotinic acid in doses of 100 mg. four times a day along with other symptomatic treatment for four days. There was slight improvement in the pigmentation of the skin. He was then given Betamide intramuscularly (20 mg. once a day) for another four days as a result of which the pigmentation disappeared and there was an improvement of the skin in general. The patient was not completely recovered when he left the hospital.

Case No. 3-K.V. female, aged 29, admitted February 24, 1942, complaining of increased frequency of stools with blood, mucus and griping (duration 6 years), pigmentation of hands, feet (duration 3 years), sourness of tongue (duration 2 months) and slight fever in the evening (duration one month). She was living on a poor diet consisting mostly of maize and wheat bread, a vegetarian, taking milk regularly, no smoking, no drinking and no other drug addiction ; she lived in a poor and congested surroundings (her husband was a peon earning Rs. 17/- p.m.).

On examination the skin over the dorsum of the hands, wrists, forearms and feet showed thickening of the epithelium keratinisation, pigmentation and at some places enfoliation leaving a red base. The feet were involved up to the ankle and the fore-arm up to the elbow joint. The medial side of the fore-arm and the area covered by the bangle was spared. The line of demarcation was very sharp. There was similar pigmentation and keratinisation round the eyes and the cheeks. Some pigmentation was present on the front and back of the neck also, cracks round the lips, the tongue was red and inflamed on both of its margins in the anterior part. In this area the papillæ were red and swollen. Gastric analysis showed no free acid. Hæmoglobin, hæmatocrit, total red blood cell count and white blood cell count were 70%, 28%, 3,970,000 per c.mm. 6,000 per c.mm. respectively. She was given nicotinic acid in doses of 100 mg. four times a day along with other symptomatic treatment as a result of which she showed remarkable progress. Pigmentation disappeared after 5 to 6 days treatment. She was discharged from the hospital in good condition on 17th March, 1942.

SUMMARY

1. The nicotinic acid content of blood and urine before and after nicotinic acid treatment was studied in 6 cases of pellagra.

It was low in both blood and urine but the decrease was more marked in urine.

2. The effect of a 'test dose' on the nicotinic acid content of blood and its excretion in urine was too small as compared with normal persons.

3. The investigation of urinary excretion may be of greater help than the blood values in the understanding of the nicotinic acid deficiency.

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THE POTENTIATION OF THE ACTION OF ADRENALINE BY EPHEDERIN ON THE SMALL INTESTINE OF RABBIT*

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The potentiation of the action of adrenaline by ephederin has been studied by various workers. Schaumann (1) found that when adrenaline in a concentration of 1 in 10 millions was added to the Ringer's solution perfusing a frog's

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legs, the addition of a similar concentration of ephederin .caused marked vaso-constriction although ephederin had no effect by itself in the absence of adrenaline. He also found that injection of ephederin increased the effect of the subsequent injection of adrenaline on the blood pressure of the rabbit and the dog. Reinitz (2) found an increase in the effects of adrenaline by ephederin on the rabbit's uterus. Pak and Tang (3) got similar results on rabbit's conjunctiva. Gaddum and Kuriatkowski (4) showed that low concentrations of ephederin sensitise the rabbit's ear, the cat's nictitating membrane and the frog's heart not only to adrenaline, but also to the stimulation of adrenergic nerves.

Experiments have now been done to prove that the action of adrenaline on the rabbit's duodenum is potentiated by ephederin in low concentrations. Ephederin prevents the destruction of adrenaline in the same way as eserine does that of acetylcholine.

METHODS

A piece of rabbit's duodenum was taken out from the animal and suspended in a bath of Tyrode solution (Nacl, 0.9g. ; Kcl, 0.042g. ; Cacl₂, 0.024g. ; Mgcl₂, 0.0005g. ; NaHCO₃, 0.05g./100 cc. distilled water). Air was bubbled all through. Temperature was maintained at about 36°C. Records of normal intestinal movements were taken. Adrenaline was then put in the bath and the changes in the tone and in the strength of contractions were observed. Subsequently the intestine was washed thoroughly and the fluid in the bath was changed. Ephederin was then put in the bath and five minutes later adrenaline was again added to give the same concentration as initially. Drugs used were Adrenaline chloride solution P. D. & Co. and Ephederin Hydrochloride B. W. & Co. solution.

RESULTS

It was observed that adrenaline in concentration ranging from 1 in 10^6 to 1 in 10^8 gave a reduction in tone and a diminution in the strength of contractions of the isolated rabbit's gut (Fig. Ia). After this record was obtained and- the gut washed free from adrenaline, ephederin in concentration of 1 in 10^6 was put in the bath. This concentration of ephederin was found most suitable for the purpose of the experiments. After ephederin had been in the bath for 5 minutes, adrenaline in the same concentration as initially was added to the bath. It was observed that adrenaline added after ephederin gave a more marked loss of tone and a greater diminution in the contractions and the contractions did not return to normal for a much longer time (Fig. Ib). Altogether 20 experiments were done in this series and the results gave the same conclusion.

Effect of adrenaline in a concentration I in 10⁷ on the contractions of the rabbit's duodenum (*a*) before ephederin (*b*) after ephederin I in 10⁶ had acted for 5 minutes. The upper tracing shows· intestinal movements, lower tracing time in 6 seconds.
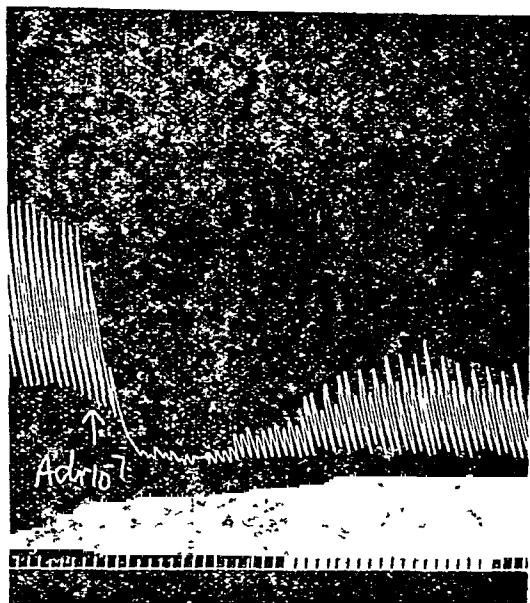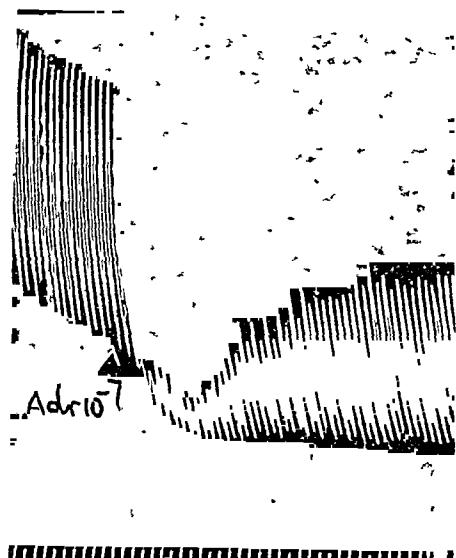
FIG. 1A   FIG 1B

It is a well established fact that the action of acetylcholine is enhanced by eserine which combines with choline esterase, the enzyme which finally destroys acetylcholine. According to Easson and Stedman the action of eserine on choline esterase is due to substrate competition. Gaddum (5) suggested that some substance allied to adrenaline in chemical structure but more stable might fill the place of eserine in experiments on adrenergic nerves. The suggestion that ephederin might act in this way came from the work of Blaschko, Richter and Schlossmann (6) who showed that ephederin is not destroyed by amineoxidase which destroys adrenaline and furthermore that in presence of ephederin this enzyme fails to destroy adrenaline. The present set of experiments show that ephederin potentiates the action of adrenaline on rabbit's duodenum and it is very probable that this potentiation is the result of slow destruction of adrenaline by the enzyme as a result of inhibition of the enzyme by ephederin.

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THE EFFECT ON RATS OF SUPPLEMENTING A POOR BENGALEE DIET WITH *l*-TYROSINE

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Some twenty-eight years back McCay (1) was of opinion that health and physique of Indian people whose diet was largely based on rice were inferior to those of peoples consuming wheat and milk due to low protein content of rice. So also Wilson and others (2) in discussing the results of a diet survey in Calcutta remarked that 'the protein element in nutrition in India may ultimately prove to be the most important and it will be the most difficult to remedy'. McCollum and his associates (3) are also of opinion that a diet based on rice as a staple requires supplementing with foods which provide proteins abundant in the essential amino acids as well as foods rich in essential mineral elements and vitamins. But Aykroyd and Krishnan (4) showed that addition of a protein like casein which consists of most of the important amino acids like lysine, histidine, tryptophan, phenylalanin and tyrosine or cystine separately which is lacking in casein, had but only slight effect in improving the nutrition of the rats on a poor Madrasee diet. These experiments in the hands of the last workers show that the poor nutritive value of an ordinary Madrasee or a Bengalee diet based on rice as a staple and consisting of pulses, vegetables, a very

small quantity of meat or fish and vegetable fat only (gingelly or mustard oil as the case may be) is most probably not due to lack of the important amino acids. But no attempt has been made, so far, by these workers to find out the usefulness of these important amino acids singly or in combination, for elaboration of essential substances like hormones in the endocrine glands, as Willcock and Hopkins (5) suggested that protein constituents may be utilized not only for tissue formation or structural maintenance but in a more specific and direct manner as for instance, in the elaboration of essential substances as adrenaline or other hormones. It was, with a view to find out the effects of *l*-tyrosine alone as a supplement of an average poor Bengalee diet, on growth and secretion of thyroid glands, that the present investigation was undertaken.

EXPERIMENTAL

Two dozen of albino rats, all females, were divided into two groups of twelve each, of average weight 59 g. The basal diet consisted of the following:—

Rice, raw polished	142 g.
Lentil	10.2 g.
Mustard oil	0.7 g.
Potatoes	7.1 g.
Palang sak	3.4 g.
Fish	0.5 g.
Common salt	1.0 g.

This diet which corresponds roughly to the daily diet of an adult of the poorer classes in Bengal was mixed and fed in proportions indicated. The first batch was having the basal diet alone whereas the second batch was having a supplement of *l*-tyrosine 0.34 g. daily for 12 rats. The animals were fed for eight weeks only on diets that differed only in their tyrosine content. The animals were occasionally exposed to the sun in the morning. The feeding experiment lasted for eight weeks during which weights of the animals were recorded weekly. Table I shows the average rate of increase in weight in the two groups during the experimental period.

TABLE I

Group.	Initial weight g.	1st. g.	2nd. g.	3rd. g.	4th. g.	5th. g.	6th. g.	7th. g.	8th week. g.
I	59	59.6	57.3	61.2	62.6	63.2	63.5	64.9	67.9
II	59	60.5	55.1	60.0	65.4	66.3	66.9	63.0	67.8

Batches of three animals from each group were kept in metabolism cages (Coonoor, type described by Niyogi and co-workers, 6), for six weeks from the second to the seventh week. Total food intake was calculated by deducting the residue left each morning from the quantity given on the previous day (dry weight). The rejected food, fæces and urine of each group were stored in separate vessels for seven days after which estimation of nitrogen in the food taken and excreta was done and the retention of total nitrogen every week was calculated. After eight weeks the animals were finally weighed, killed under ether anæsthesia and the thyroid glands were taken out for histo-pathological examination.

The intake, excretion and retention of nitrogen in the two groups of animals for six weeks are given in Table II.

TABLE II

Experimental week.	Group.	Total N_2 in food taken. g.	N_2 in fæces. g.	N_2 in urine. g.	Total N_2 in the excretion. g.	Total N_2 retention g.
2nd week	I	2.994	0.479	0.791	1.270	1.724
	II	2.724	0.460	0.870	1.330	1.394
3rd week	I	3.088	0.516	0.910	1.456	1.632
	II	3.226	0.549	1.111	1.660	1.566
4th week	I	3.282	0.590	0.793	1.383	1.899
	II	3.593	0.610	0.680	1.290	2.303
5th week	I	2.904	0.445	0.787	1.232	1.672
	II	3.345	0.435	1.005	1.140	1.905
6th week	I	2.981	0.556	0.787	1.343	1.611
	II	2.896	0.513	0.931	1.447	1.449
7th week	I	3.184	0.556	0.630	1.186	1.998
	II	3.112	0.319	0.396	0.715	2.127

Histological examination of the thyroid glands of the two groups of animals showed the following structure respectively:—
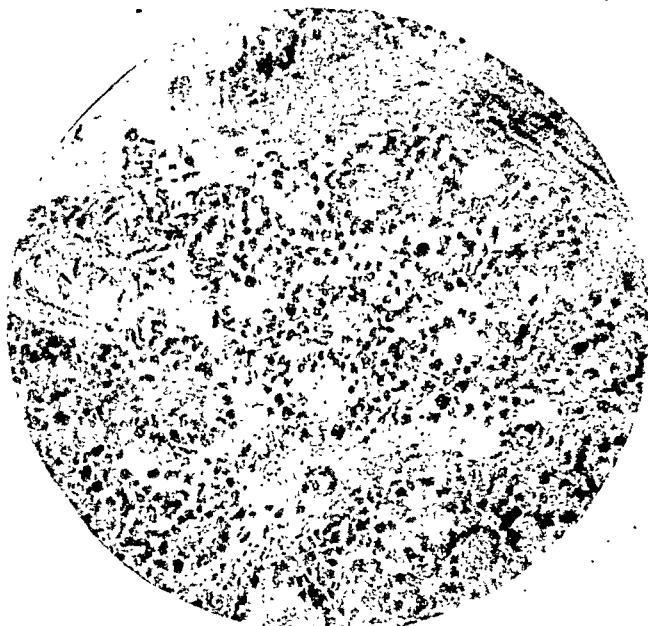
PLATE I

Showing hyperplastic condition (with fibrosis) of the thyroid gland as a result of feeding of the poor-Bengalee diet.
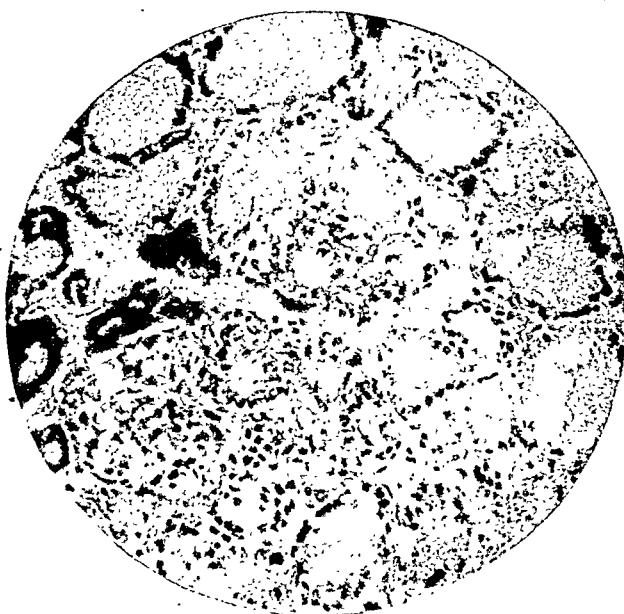
"PAL & BOSE"

PLATE 2

Showing normal structure of the thyroid gland, as a result of addition of *l*-tyrosine to the poor-Bengalee diet.

"PAL & BOSE"

Glands of the animals belonging to Group I—The alveoli are not clearly indicated and there is proliferation of the alveolar epithelium without showing much colloid material inside the visicles. There is also a good deal of increase in fibrous tissue inside the gland. The entire structure is characteristic of a hyper-plastic condition of the glands.

Glands of the animals belonging to Group II—The alveoli are well defined, and most of the visicles that are full of colloid material are lined by thin and flattened cubical epithelium. Some of the visicles are, however, empty and some vacuolated, showing moderate activity on the part of the glands. The structure is more or less normal.

DISCUSSION

Table I that records the progress in weight of the two groups of animals shows that addition of *l*-tyrosine to a typical poor Bengalee diet has no supplementing effect so far as growth is concerned. This is quite contrary to Abderhalden's (7) early studies indicating that tyrosine is a nutritive essential but is quite in accordance with the findings of Totani (8) that in the case of rats most of the individuals fed upon a dietary, which contained at most a minimal supply of tyrosine, not only maintained their body weight but also exhibited growth ; and of Lightbody and Kenyon (9) that growth of young rats over a period of twelve weeks was independent of the tyrosine content of the diet. But Womack and Rose (10) who also performed similar experiments, did not consider that their results gave the final proof that tyrosine was not an indispensable amino acid but pointed out that if it be an essential dietary constituent, the quantity required must be very small, since the daily intake of tyrosine of their rats could not have exceeded 2.1 mg. The last mentioned workers subsequently (11) showed that tyrosine deficiency alone permitted good growth and its addition to the diet provided no indication that this amino acid was needed for growth. Our results were quite in accord with these observations so far as growth was concerned.

Abderhalden (*loc. cit.*) on feeding rats on amino acid mixture free from *l*-tyrosine found that the nitrogen balance immediately became negative. Upon addition of tyrosine or phenylalanine to the amino acid mixture the nitrogen balance rose although equilibrium was not attained. The nitrogen metabolism experiment in our hands (*vide* Table II) did not show higher retention of nitrogen always by animals whose diet was supplemented with *l*-tyrosine. For second, third and sixth weeks group II showed less retention of nitrogen, whereas for the other three weeks in a total period of six weeks during which the metabolism experiment was conducted the same group showed a rise in the nitrogen balance as compared to group I. So here too our findings are contrary to Abderhalden's observations regarding the nitrogen balance, on addition of *l*-tyrosine to the diet.

But difference in the histological structure of the thyroid glands of the animals belonging to the two groups, shows definitely that addition of *l*-tyrosine to the ordinary Bengalee diet which is very much deficient in this important amino acid, is necessary for keeping the structure of the glands healthy and normal,

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which otherwise exhibits a state of hyperplasia, characteristic of abnormal hyper-activity on the part of the glands. So although it has been proved definitely by Totani, Lightbody and Kenyon, and Womack and Rose (*loc. cit.*) that tyrosine is not an indispensable amino acid for growth, Willcock and Hopkins (*loc. cit.*), are of opinion that some amino acids may be not only utilized for tissue formation of structural maintenance but also in a more specific direct manner for elaboration of essential substances like hormones. Similarity of constitution between tyrosine and thyroxine has been amply proved and even synthesis of thyroxine has been effected by Harrington and Barger (12, 13) and according to Lloyd· and Shore (14) di-iodotyrosine and thyroxine are the only two iodine containing compounds of thyroid with equal distribution of iodine among them. The latter authors also consider tyrosine and phenylalanine as of particular interest as the probable precursors of di-iodotyrosine, thyroxine and adrenaline. The histological structure of the thyroid glands of the two groups of animals on tyrosine deficient diet and on the same basal diet supplemented by the amino acid respec-tively, supplies ample proof of the essential nature of *l*-tyrosine so far as thyroid secretion is concerned although its indispensable nature has been questioned from a pure and simple nutritive point of view.

SUMMARY

1. *l*-tyrosine has no supplementing effect on ordinary Bengalee diet. Its addition to the diet provides no indication that this amino acid is needed for growth.

2. Nitrogen balance is also not much affected by addition of *l*-tyrosine to the average poor Bengalee diet.

3. But most probably it is indispensable so far as the secretion of the thyroid glands is concerned. The deficiency of the amino acid leads to histo-pathological changes.

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COMPARATIVE STUDIES OF POTASH ALUM AND FERRIC ALUM
AS ADJUVANT TO DIPHTHERIC TOXOID

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INTRODUCTION

Glenny *et al* (1) used alum (potassium aluminium sulphate) for precipitating diphtheria toxoid and noted increased antigenic efficiency shown by the precipitate. Wallace (2) used 1% Ferric alum successfully in precipitating diphtheria toxin. Smith (3), after trying different metallic salts, considered potash alum to be the reagent of choice for use with diphtheria toxoid. She did not try ferric alum. Eaton and Gronau (4) observed that purified diphtheria toxin is readily precipitated by ferric alum between pH 6 and 7 and tetanus toxin is precipitated almost completely with the same salt.

Prolonged use of Potash alum. with diphtheria toxoid for hyper-immunisation of horses in the manufacture of therapeutic sera has been observed to give rise to marked deterioration of general health of the animals causing chronic constipation, emaciation, lassitude, loss of appetite and damage to liver and kidney. Suspensions obtained on adding ferric ammonium sulphate and potassium aluminium sulphate to diphtheria culture filtrates were studied side by side 'in vitro' and 'in vivo' with a view to find out whether the ferric alum can be used as a substitute for potash alum for animal immunisation with advantage.

EXPERIMENTAL

Diphtheria toxin was prepared in veal infusion broth of pH 7.8 containing 2% lactopeptone and 0.5% sodium chloride. The Park William 8 strain was used.

To 4 different fractions from each lot of toxin ferric alum and potash alum sufficient to give 1% and 2% strengths of each were added. The precipitates obtained were washed thrice and then suspended in normal saline added upto the original volume of toxin. Small quantities of sodium citrate were then added

to each and the samples kept in water bath at 45°C till clear solutions were obtained. These were tested by Ramon's flocculation method. 10 different lots of toxin were studied. With 1% potash alum average recovery of antigen was found to be 44% of original, with 1% ferric alum 53%, with 2% potash alum 71% and with 2% ferric alum 85%.

Two samples of diphtheria toxin were completely detoxified with formalin. To two different fractions of each ferric alum and potash alum sufficient to make 0.5% strength in the mixtures were separately added and pH brought to 7 with 20% solution of sodium carbonate. Volume was then so adjusted with normal saline that 2.25 cubic centimetres of the suspensions contained 2 cubic centimetres of the original toxoids. 2.25 cubic centimetres quantities from the four suspensions were injected subcutaneously into each of four groups of guinea-pigs—each group consisting of six animals. Six weeks later the pigs were bled from heart and the antitoxin contents of the sera obtained were separately determined intra-dermally on guineapigs. The results obtained are shown in Table I.

TABLE I

Lot number of toxoid.	Adjuvant added to toxoid.	Average of titres obtained in six guinea-pigs. (unit per cubic centimetres of serum).
565	0.5% Ferric alum	0.15
	0.5% Potash alum	0.28
579	0.5% Ferric alum	0.18
	0.5% Potash alum	0.3

SUMMARY

It appears that when medium described in the text is used for culture, ferric ammonium sulphate precipitates diphtheria toxin from filtrates more effectively than potassium aluminium sulphate. But suspensions obtained with ferric alum is less antigenic 'in vivo' than that obtained with potash alum.

My thanks are due to Dr. B. N. Ghosh, D.Sc., for his valuable suggestions.

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INVESTIGATIONS ON THE NEW ANTIDIABETIC PRINCIPLE (AMELLIN) OCCURRING IN NATURE. PART III. ITS INFLUENCE IN INCREASING HÆMATOPOITIC ACTIVITY IN DIABETICS

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It has long been recorded that some defect in the oxygen transforming system from any cause whatsoever has direct or indirect influence in impairing various normal functions of the organism ; and this type of disturbance has been suggested by Bodanskey and Bodanskey (1) to have a tendency to hamper the normal production of hæmoglobin and red blood cells.

Castle and his associates (2) have been able to show that gastric mucosa and gastric juice of patients suffering from pernicious anæmia are deficient in a substance which is present in normal individuals and which controls the maturation of red cells in the bone marrow. The generally accepted view is that, it is this principle designated the "intrinsic factor" which interacts under normal condition during digestion with an "extrinsic factor" present in the food protein and gives rise to the antianæmic or hæmatinic substance which is mainly stored in the liver. Wilkinson and Klein (3) reported isolation of an active principle "hæmopoitin" from gastric juice and suggested this to be same or allied with the intrinsic factor of Castle. Goodall (4) has, however, shown that transfusion of blood from normal individuals in case of pernicious anæmia, causes great influence in bringing the colour index to the normal limit. Acute hæmolytic anæmia of Lederer (5) which occurs suddenly and causes gastro-intestinal troubles as well as enlargement of liver, spleen etc. and which was considered to be infectious in origin, has also been found to have great response to blood transfusion therapy. All these do not seem to be without significance and indicate the possibility of the existence of some factors within the blood of normal individuals, deficiency of which might have something to do with the onset of troubles. Bodanskey and Bodanskey

(*loc. cit.*) suggested that transfusion of blood exerts an antilytic action and stimulating influence on the liver in producing antianæmic-red cell maturation principle. Its action by checking etiological infectious agents has also been suggested. A direct evidence of this conception has recently been found from the work of Joseph (6), who observed the presence of an antihæmolytic factor in normal human-plasma.

A good response to marmite has been observed by Goodall (*loc. cit*) and Napier and Majumdar (7) in case of macrocytic anæmia of pregnancy. They observed similar blood picture just like that obtained by Wills (8) who noticed the absence of the Van den Bergh test as an important distinguishing feature between the anæmia described by her and the pernicious anæmia where the test was positive. Napier and Majumdar (*loc. cit.*) have further suggested that the special hyperchromic anæmia as observed by them might be due to a conditional deficiency in important food factors the detailed nature of which is still unknown. According to them "this deficiency in pregnancy is conditioned by the presence of the fœtus with its extra demands for substances essential for hæmopoisis (or less probably its toxic influence)."

Apart from the consideration of specific deficiencies of substances possessing hæmatopoitic activity, records have also been made of inhibition of bone marrow activity by toxic agents, external as well as internal. Hamilton (9) showed the effect of benzene in depressing the bone marrow activity, when rate of production of blood can not keep pace with its rate of destruction, thus leading to anæmia. Toxins generated internally in case of chronic infections may have also some influence either in neutralising the hæmatopoitic principle within the system or in inhibiting the bone marrow activity.

Motzfeldt (10), on the other hand, discussed how diabetes mellitus may be of hepatic origin in which case insulin is only of slight value. Roy and Mukherjee (11) as well as Mirsky *et al* (12) have shown that the metabolic disturbance in diabetes is due to an alteration in the balance between the hepatic glycogenesis and glycogenolysis. The recent findings of Roy (13) that hyper-chlorohydria and resulting acidosis are at the root of onset of hyperglycemia in most cases, have created great sensation in this field of research. Some such postulation was also made by Elias (14) in 1912, when he succeeded in producing hyperglycemia in dogs and rabbits with hydrochloric acid.

It is thus evident that disturbed condition of the stomach as well as of liver which is responsible for the production of hyperglycemia in most cases, may also be the causative factor for anæmia in those patients.

While continuing the series of works on the physiological influence of the new antidiabetic principle, recently reported from this laboratory (15, 16), it was observed that the patients who had been suffering for a pretty long time, looked anæmic, characterised by development of pallor and weakness, purpura and even

retinal hæmorrhages in some cases. Along with the reduction of hyperglycemia we found, to our utter surprise, marked improvements regarding this point which suggested planning of counting red blood cells and estimating hæmoglobin percentage in the blood of the patients at different stages of their recovery. Moreover, it has been suggested by Nath (17) that a deficiency of a type of substance related to the antidiabetic principle, amellin, is most probably responsible for the onset of hyperglycemia in the long run. All these prompted the authors to look into the matter more thoroughly and to see if ingestion of amellin could, in any way, bring relief in anæmia associated with diabetes.

EXPERIMENTAL

According to the findings of Osgood, Haskins and others, Kolmar and Boarner (18) recommend using oxalated blood for hæmoglobin estimation and red cell count within twenty-four hours after collection.

Counting of Red Blood Cells

Enumeration of the red blood corpuscles was made in the usual way by Thoma Zeiss Hæmocytometer using Hayem's diluting fluid which is of the following composition.

Sodium sulphate	...	...	...	5.0 gms.
Mercuric chloride	...	...	...	0.5 gms.
Water to make	...	...	...	200.0 cc.

Estimation of Hæmoglobin :

Colorimetric estimation of iron in the blood was made according to the method of Wong (19) and Hæmoglobin in gm. per 100 cc. of blood calculated from the equation:

$$\frac{\text{Iron in mg. per 100 cc.}}{3 \cdot 35} = \text{Hæmoglobin in gms. per 100 cc. blood.}$$

The method consists in detaching iron from the hæmoglobin molecule by treatment with sulphuric acid in the presence of potassium persulphate without heating and in removal of proteins by tungstic acid, and determining it in the filtrate colorimetrically by the thiocyanate reaction.

Colour Index :

It has long been known that in pernicious anæmia, the colour index of the blood, which signifies the relative hæmoglobin content of the average corpuscles, becomes high. Sometimes it may reach the value 1.6 or more, while in case of normal individuals it should be about 1.0.

This can be easily calculated from the results of Hb (gms.) per 100 cc. and R.B.C. count (million) per cubic millimeter according to the following formula : —

Colour index (C.I.)=

$$= \frac{\text{Hb. (gms.) per 100 cc. blood}}{\text{R.B.C. No. (Million) per c.m.m.}} \div \frac{x}{y}$$

where x means mean gms. of hæmoglobin per 100 cc. of normal blood and y means mean normal erythrocytes number in millions per cubic millimeter.

Values of x & y have been taken as 15.0 and 5 respectively as suggested by Haden (20). Colour Index can also be calculated according to Osgood (21) by dividing gms. of Hb per 100 cc. of blood by Hb-co-efficient, which is 14.7 for males and 14.3 for females.

It will be seen from the results shown in the following tables that the colour indices of the diabetics suffering for a pretty long time were very high initially which came down to the neighbourhood of 1.0 through ingestion of amellin.

Iron Index :

It has been proposed by Murphy, Lynch and Howard (22) that iron index is the figure obtained by dividing the mgms. of iron in 100 cc. of blood by R.B.C. in millions per c.m.m. and the average value under normal condition was found to be 10.73 and 9.74 for males and females respectively. This index is known to be elevated in macrocytic hyperchromic anæmia and greatly reduced in microcytic hypochromic anæmia. Calculation of iron indices of our diabetic patients at different stage of their recovery was also supposed to yield interesting information regarding the nature of anæmia from time to time.

Before discussing the results obtained by clinical studies it is necessary to give the introductory note regarding the patients. Detailed informations of 15 patients have already been given in the previous part (16) which need not be mentioned once again, particulars of 9 more patients whose results will also appear in this part are shown in Table I.

Detailed results with one patient showing reticulocytic response in addition to the reduction of hyperglycemia and other symptoms of relief in anæmia are shown in Table II. Clinical results obtained with all the patients along with the calculated colour indices etc. are shown in Table III.

This may be recorded here that results with actual days of treatment are represented in the figures 1 & 2 while the calculation of days have been done in terms of months and half month in Table III for accommodating all the data with different patients in a condensed form.

TABLE I

Pt. No.	Age in years.	Sex and Religion.	Profession.	Duration of disease.	When treatment began.	Urine Sugar (24 hrs. excretion)		Total calories intake.	Body weight. lb.
						Initial. %	Present. %		
P5	75	Male, Hindu	Retd. Govt. Officer	25 years.	4.12.41	1.5	Nil.	2877	154
P13	69	Do.	Nayeb of Zaminder's State		18.3.42	7.1	Nil.	3121	112
P16	48	Do.	Teacher Govt. School	12 years.	21.8.42	1.2	Nil.	1822	129
P17	52	Do.	Medical Officer	2 years.	21.8.42	3.3	0.5	1851	130
P18	18	Do.	Head Master	4 years.	9.9.42	1.5	Nil.	2840	114
P19	70	Do.	Businessman	8 years.	17.10.42	3.5	1.5	2502	122
P20	43	Do.	Merchant	1 year.	26.11.42	1.5	Nil.	1776	132
P21	38	Male, Muslim	Head Master Govt. School	3 years.	22.12.42	1.7	0.5	2000	132
P22	39	Male, Hindu	Zaminder	8 years	9.1.43	1.9	...	2465	182
P23	44	Do.	Munsiff	2 years.	18.1.43	1.92	Nil.	2819	122
P24	67	Do.	Retd. Head Master	3½ years.	20.2.43	0.8	Nil.	1900	132

TABLE II

Blood picture of a diabetic (P₅) showing reticulocytic response to amellin.

Day of treatment.	Blood sugar mgms/100 cc.	Iron mgms /100 cc.	Hb, gms/ 100 cc. (calculated)	R.B.C. in million/ c.m.m.	Colour index.	Iron Index.
0	264.0	77.1	23.02	...	...	...
12	285.6	83.8	25.01	4.64	1.79	18.06
27	329.4	53.39	15.93	4.3	1.23	12.41
43	298.8	61.2	18.27	4.13	1.47	14.8
56	309.8	58.39	17.42	5.08	1.14	11.4
71	304.1	51.7	15.44	4.23	1.21	12.2
86	296.5	61.73	18.43	5.07	1.21	12.1
107	283.8	54.94	16.40	4.94	1.10	11.1
120	271.6	51.01	15.23	4.18	1.21	12.2
135	268.0	...	...	4.84	...	...
176	270.0	50.3	15.02	5.0	1.00	10.06
195	264.0	51.2	15.29	5.1	0.99	10.03
265	180.4	52.4	15.64	4.7	1.1	11.1
290	148.0	44.6	13.31	4.3	1.02	10.3
305	139.0	45.4	13.55	4.4	1.01	10.3

TABLE III

Period of treatment in months.	Patient No. 2				Patient No. 3			
	B.S.	Fe.	R.B.C.	C.I.	B.S.	Fe.	R.B.C.	C.I.
0.0	162.0	...	...	...	297.2	...	...	...
0.5	142.1	...	...	...	266.0	...	...	...
1.0	120.5	...	...	...	...	...	...	...
1.5	...	...	...	...	...	...	...	...
2.0	...	...	...	...	206.0	...	3.02	...
2.5	...	...	...	...	180.0	69.74	...	...
3.0	228.0	...	...	...	198.2	75.18	3.57	2.08
3.5	...	67.34	...	...	184.8	...	3.63	...
4.0	214.0	61.85	...	...	212.2	51.74	4.71	1.09
4.5	221.5	...	5.01	...	...	...	...	...
5.0	191.3	62.0	5.69	1.08	185.0	47.47	4.62	1.02
5.5	...	...	...	...	...	...	...	...
6.0	193.4	58.36	5.08	1.14	172.0	49.15	5.16	0.94
6.5	153.7	52.05	5.92	0.87	160.0	52.54	4.82	1.08
7.0	159.5	58.77	5.99	0.97	150.0	45.25	4.80	0.94
7.5	155.6	61.63	5.13	1.13	162.0	50.2	5.0	0.99
8.0	135.0	52.16	4.81	1.08	...	...	...	...
8.5	131.0	59.3	4.92	1.19	125.0	47.2	4.9	0.95
9.0	...	...	...	...	120.0	...	5.2	...

TABLE III—(Contd.)

Period of treatment in months.	Patient No. 4				Patient No. 5			
	B.S.	Fe.	R.B.C.	C.I.	B.S.	Fe.	R.B.C.	C.I.
0.0	176.0	...	...	...	264.0	77.1	...	...
0.5	...	...	...	...	285.6	83.8	4.64	1.79
1.0	...	...	...	...	329.4	53.39	4.3	1.23
1.5	...	...	...	...	298.8	61.2	4.13	1.74
2.0	162.0	...	...	...	309.8	58.3	5.08	1.14
2.5	...	...	...	...	301.1	51.7	4.23	1.21
3.0	128.0	62.3	5.01	1.23	296.5	61.73	5.07	1.21
3.5	122.6	67.28	5.33	1.25	283.8	54.94	4.94	1.01
4.0	121.0	...	6.27	...	271.6	51.01	4.18	1.21
4.5	130.0	59.23	5.99	0.99	268.0	...	4.84	...
5.0	126.0	60.73	5.96	1.01	...	...	...	...
5.5	...	...	...	...	...	...	...	...
6.0	127.0	57.48	5.49	1.04	270.0	50.3	5.0	1.01
6.5	125.0	54.36	5.03	1.07	264.0	51.2	5.1	0.99
7.0	120.0	...	4.74	...	...	...	...	...
7.5	115.0	50.3	5.1	0.96	165.0	...	5.1	...
8.0	110.0	53.7	5.6	0.94	...	...	...	...
8.5	...	...	...	...	...	...	4.7	...
9.0	...	...	...	...	180.0	52.4	4.3	1.10
9.5	...	...	...	...	148.0	54.6	4.4	1.02
10.0	...	...	...	...	139.0	45.4	...	1.01

Period of treatment in months.	Patient No. 7				Patient No. 8			
	B.S.	Fe.	R.B.C.	C.I.	B.S.	Fe.	R.B.C.	C.I.
0.0	235.9	71.24	6.49	1.09	314.3	52.08	4.5	1.15
0.5	...	...	...	...	258.8	54.36	5.71	1.04
1.0	244.5	60.94	6.25	0.97	271.6	49.71	5.0	0.99
1.5	209.6	67.06	5.41	1.23	...	...	...	...
2.0	...	...	...	...	...	...	...	...
2.5	...	...	...	...	252.0	54.7	6.4	1.0
3.0	177.8	61.86	5.6	1.15	...	...	...	...
3.5	169.2	62.12	5.63	1.09	252.6	59.4	5.35	1.09
4.0	162.0	...	...	...	242.0	...	...	...
4.5	...	...	...	...	...	...	...	...
5.0	...	...	...	...	235.0	59.5	5.25	1.18
5.5	...	...	...	...	...	...	...	...
6.0	180.0	56.0	5.6	0.99	...	...	...	...
6.5	...	...	...	...	...	...	...	...
7.0	...	...	...	...	217.0	48.7	4.45	1.08
7.5	...	...	...	...	...	...	...	...
8.0	...	...	...	...	210	...	...	...
8.5	...	...	...	...	...	...	...	...
9.0	...	...	...	...	221.0	47.6	4.2	1.12
9.5	...	...	...	...	...	...	...	...
10.0	...	...	...	...	...	...	...	...

TABLE III—(Contd.)

Period of treatment in months.	Patient No. 9				Patient No. 13			
	B.S.	Fe.	R.B.C.	C.I.	B.S.	Fe.	R.B.C.	C.I.
0.0	263.5	49.2	3.88	1.26	248.6	63.6	4.31	1.47
0.5	216.4	56.8	4.04	1.39	226.0	53.6	4.25	1.25
1.0	200.2	46.5	4.2	1.10	218.0	...	5.88	...
1.5	...	...	...	...	...	...	...	...
2.0	184.2	48.1	4.48	1.07	205.0	50.1	5.2	0.95
2.5	...	...	...	...	...	...	...	...
3.0	191.0	...	4.96	...	206.0	...	...	...
3.5	...	...	...	...	...	...	...	...
4.0	173.0	...	5.3	...	218.1	...	...	...
4.5	176.4	...	...	...	...	...	...	...
5.0	...	...	...	...	...	...	3.84	...
5.5	165.0	39.0	4.1	0.94	...	...	...	...
6.0	...	...	...	...	216.9	53.5	...	1.38
6.5	...	...	...	...	...	...	...	...
7.0	121.5	52.02	3.86	1.15	165.0	40.7	4.03	1.0
7.5	...	...	...	...	...	...	...	...
8.0	115.6	38.0	3.5	1.05	164.1	51.5	3.94	1.2
8.5	...	...	...	...	...	...	...	...
9.0	...	...	...	...	160.0	47.1	4.3	1.05
9.5	...	...	...	...				
10.0	121.0	44.1	3.9	1.11	155.0	50.7	4.35	1.15
11.0	100.0	38.1	4.1	0.91	116.0	51.02	5.20	0.95

Period of treatment in months.	Patient No. 10				Patient No. 11			
0.0	239.0	60.05	4.6	1.3	121.0	35.13	2.16	1.62
0.5	242.2	58.48	5.0	1.16	120.2	31.86	3.0	1.05
1.0	205.0	55.4	4.78	1.15	113.0	...	4.01	...
1.5	...	...	...	...	...	...	...	...
2.0	198.0	...	4.9	...	108.0	37.3	3.62	1.02
2.5	202.0	...	5.4	...	...	...	...	...
3.0	183.0	56.0	5.6	0.99	85.0	44.45	4.3	1.02
3.5	...	...	...	...	...	...	...	...
4.0	...	...	...	...	83.0	...	4.6	...

Period of treatment in months.	Patient No. 14				Patient No. 15			
0.0	48.3	...	5.6	...	223.0	65.3	5.42	1.19
0.5	62.6	54.18	5.42	1.03	228.0	...	...	...
1.0	...	...	...	...	216.0	...	5.6	...
1.5	...	...	...	...	...	...	...	...
2.0	...	...	...	...	216.0	57.9	5.36	1.07
2.5	...	...	...	...	213.8	55.38	5.5	...
3.0	85.0	58.2	5.7	1.01	...	...	...	...
3.5	...	...	...	...	...	...	...	...
4.0	84.0	56.0	5.6	0.99	...	...	...	...

TABLE III—(*Contd.*)

Period of treatment in months.	Patient No. 16				Patient No. 17			
	B.S.	Fe.	R.B.C.	C.I.	B.S.	Fe.	R.B.C.	C.I.
0	140.4	...	...	...	293.0	65.2	4.1	1.55
1	126.8	...	...	...	255.8	50.25	4.16	1.2
2	...	...	...	...	...	...	...	...
3	116.3	50.2	3.56	1.4	298.0	70.4	5.3	1.3
4	103.0	58.9	4.87	1.19	265.0	47.05	4.2	1.11
5	121.6	50.0	4.31	1.15	238.6	45.02	3.9	1.37
6	100.0	47.6	4.09	1.15	248.5	52.08	4.4	1.17
7	...	...	...	...	225.0	58.1	4.9	1.17
8	...	...	...	...	233.6	55.2	4.8	1.13

	Patient No. 18				Patient No. 19			
0	242.0	...	...	...	256.0	55.4	...	...
1	224.0	...	...	...	...	...	...	...
2	...	...	...	...	202.0	60.8	4.1	1.48
3	202.3	66.2	4.5	1.46	202.0	59.3	3.9	1.51
4	125.0	50.2	3.9	1.27	127.6	58.1	4.38	1.34
5	...	...	...	...	182.0	56.8	4.59	1.22
6	98.0	52.3	4.4	1.18	183.0	59.5	4.9	1.20
7	96.0	...	...	...	191.0	46.5	5.35	0.85

	Patient No. 20				Patient No. 21			
0	189.3	50.7	4.2	1.2	223.0	57.1	4.1	1.38
1	98.0	52.6	4.4	1.18	222.0	55.8	4.41	1.25
2	...	...	...	...	225.0	59.5	4.7	1.25
3	123.0	51.2	4.5	1.12	190.0	54.5	4.9	1.01
4	105.0	51.8	4.6	1.11	168.0	60.6	4.88	1.2
5	105.0	61.7	4.98	1.2	...	...	...	...

	Patient No. 23				Patient No. 21			
0	180.0	48.5	4.3	1.12	147.0	66.6	4.9	1.31
1	162.0	50.0	3.92	1.26	97.0	49.01	4.05	1.2
2	156.0	51.1	4.2	1.2	70.0	51.5	4.47	1.1
3	151.0	52.3	5.4	0.96	75.0	51.6	4.5	1.2

N.B. B.S. represents sugar in mgms. per 100 cc. blood; Fe represents mgms. of Iron per 100 cc. blood; R.B.C. represents red blood cells in millions per c.mm. and C.I. represents the colour index.

FIG. 1

FIG. 2

FIG. 3
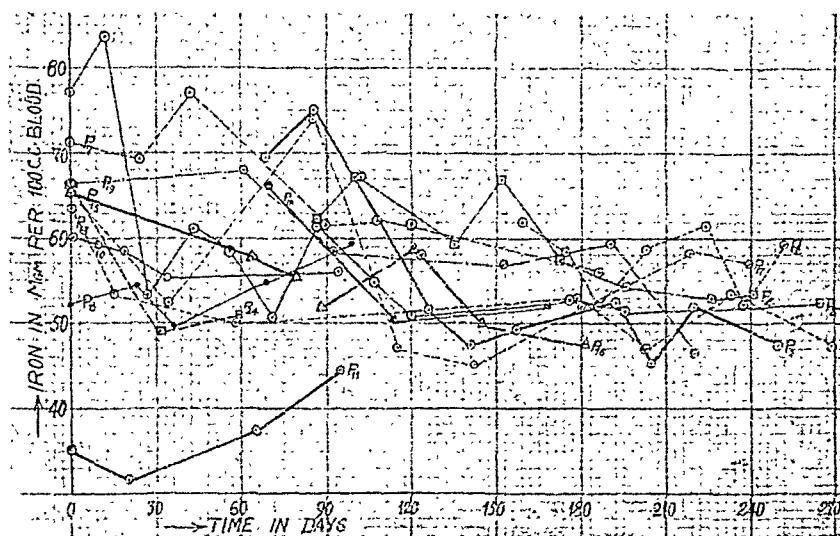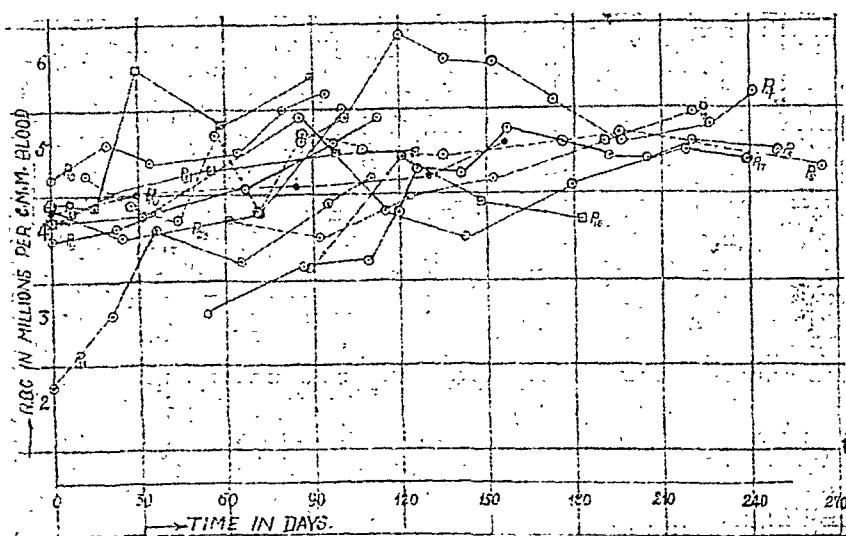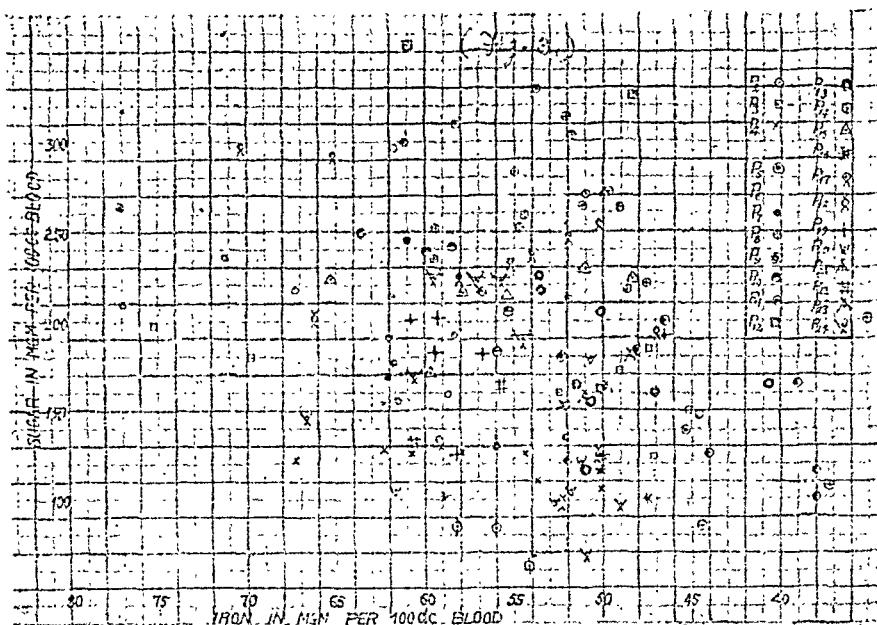
Discussion of Results

The most striking fact which is revealed from the analysis of blood of diabetics for iron, is that, almost in all the cases there are obtained initial high values of iron indicating the possibility of accumulation of nonhæmoglobin cellular iron in the blood. Jenkins and Thomson (23) have shown recently that this fraction of iron is markedly elevated in pernicious anæmia, aplastic anæmia and in some cases of anæmia resulting from chronic loss of blood (piles etc.). Average size of the red cells in case of some diabetics suffering for a considerably long period was also found by us to be increased to a great extent thus showing the symptoms of "Macrocytic Anæmia". The colour index in some case was also as high as 1.70 or more. This is to be noted that values as high as 1.6 were observed in diabetics by Lawrence, Lucus and McCance (24). It was through the administration of amellin that we observed definite improvements in these symptoms of anæmia characterised by fall in iron content of the blood to the normal value, rise in reticulocyte count, and fall of colour index to 1.0.

An interesting phenomenon was noticed almost in all cases, particularly with those suffering for a pretty long time. The iron content in the blood of those patients, though initially found to be very high, (sometimes 83.8 mg. per 100 cc.), easily fell, to our utter surprise, to the range of normal limit within the period of only one or two month's course or so of using amellin (fig. 1.) ; and this value did never rise back to the initial higher level even in spite of the

fact that the reduction in hyperglycemia was not so pronounced within that small period. In some cases, however,—particularly in case of those patients who stopped taking insulin and began using amellin—there was slight rise in blood sugar during the first few weeks ; but the iron percentage of blood reached the normal limit soon.

It can be seen easily from the Fig. 3, which represents relative change of iron and sugar in blood of diabetics, during the course of administration of amellin, that all the figures for iron in blood of all the patients collected after allowing them to take amellin for one or two months only, happen to come within the limit of 47 to 62.5 mgs. per 100 cc. ; the mean value being 53.75. This value of iron corresponds to 16.03 gr. of hæmoglobin per 100 cc. of blood ; and it may be noted that this almost coincides with the mean value with normal healthy individuals as laid down by others or observed by us. The abnormally high percentage of iron in the blood of diabetics may owe its origin to the high proportion of vegetables in their diets which are very rich sources of iron. The elevation in the iron content is also indicative of defective iron metabolism.

No definite relationship could be established between blood sugar and iron content in blood. It will be seen from figure 3 that values of iron in some case (P$_5$) after a particular "adjusting period" oscillated within the normal limit (45 to 62.2 mg.), though the amount of sugar in the blood was still exceeding 300 mg. per 100 cc.

Iron indices of patients which were in the neighbourhood of 20 or so (about double the normal value) also came to the normal limit very soon.

It can be noted from Table III and fig. 2 that patients, who had very low figure for R.B.C. initially, showed excellent reticulocytic response and the number reached the normal limit (i.e. from 4.1 to 5.8 millions per c.m.m.) in course of a few weeks' time.

Though it is too early to suggest that either the intrinsic or extrinsic food factor or the general hæmatopoitic principle formed in combination with these or any other components may be in some way or other related to the antidiabetic principle amellin, it can be laid down with certainty that it has influence on hæmatopiosis in diabetics.

All these findings may also lead to some insight into the mechanism of the action of amellin in diabetics which may be as follows:—

First the disturbed condition of blood regarding the distribution of iron in diabetics is set right which might cause utilisation of the non-hæmoglobin iron and thus bring the value of iron to the normal range. When this is achieved, fall in blood sugar commences slowly but gradually. It is possibly in this way that the real cause of trouble, which may be associated with the faulty condition of blood, is removed and better utilisation of carbohydrate results.

Further work which is in progress will, it is hoped, throw more light into the matter.

SUMMARY

1.   Hæmatopoitic effect of amellin on twenty-four diabetics has been recorded.

2.   Amellin has been found to cause utilisation of iron in the blood of diabetic patients thus bringing the abnormally high value to the normal limit within about a month or so.

3.   Great reticulocytic response to amellin in diabetics initially having low R.B.C. count, has also been observed.

4.   The principle of amellin action has been suggested to be different from that of insulin. Amellin accelerates the metabolism of iron in diabetics which occurs in excess and the reduction in hyperglycemia takes a definite course only after the expiry of such initial "adjusting period". This adjusting period may be different in different patients varying with duration and intensity of the disease.

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INVESTIGATIONS ON THE NEW ANTIDIABETIC PRINCIPLE (AMELLIN) OCCURRING IN NATURE. PART IV. ITS EFFECT ON THE UTILISATION OF INORGANIC PHOSPHATE IN THE BLOOD OF DIABETICS

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It has long been recorded by Harden and Young (1, 2) that phosphates accelerate fermentation of sugar by yeast. They also discovered the presence of hexose-di-phosphoric acid as one of the intermediate products formed during the course of reaction thus indicating the nature of metabolism of hexose. The occurrence and isolation of such esters has also been reported by Neuberg (3), Rabison (4), Embden and associates (5) and several other investigators, and it has been established that defective carbohydrate metabolism, brought about by

any factor whatsoever, is accompanied with decrease in the esterification of hexose with phosphoric acid and results into the gradual accumulation of inorganic phosphate in the blood.

Bose and De (6) have shown recently that there exists a close relationship between the carbohydrate metabolism and inorganic phosphate of the blood and both injection of insulin, and ingestion of glucose by mouth cause lowering of its inorganic phosphate content in normal as well as diabetic subjects. Similar fall in the inorganic phosphate content of blood by glucose ingestion has been shown by Blatherwick, Buel and Hill (7) and several others. Intravenous injection of glucose has also been shown to bring about fall in the inorganic phosphate by Pijoan and Quigley (8).

Amellin, which has recently been found by Nath and Banerjee to cause reduction in the hyperglycemia (9), might exert its influence by bringing about esterification of hexose with phosphoric acid which would be revealed from the estimation of inorganic phosphate in the blood of diabetics at different stages of progress and the present investigations were undertaken to throw some light on the matter.

EXPERIMENTAL

Estimation of inorganic phosphate in the blood was made according to the method of Brigg (Harrison's Chemical methods in clinical medicine, p. 365) which comprises of

(a) Precipitation of proteins in a measured volume of blood with 20% trichloro-acetic acid,

(b) Treatment of an aliquot part of the protein-free filtrate with molybdic acid and reduction of the resulting phospho-molybdate by hydroquinone in presence of acid and sodium sulphite,

(c) Estimation of phosphate colorimetrically against a standard phosphate solution treated under identical condition.

Estimation was made within half an hour after the collection of blood. Blood sugar was estimated simultaneously according to the method of Hagedorn and Jensen (10).

Results with 12 diabetic patients, the case history of whom has already been reported in the previous part (11), are shown in Table I (a) and (b) and represented graphically in fig. 1. Blood sugar values are also shown side by side. It was thought desirable to find out the average figure for inorganic phosphate in the blood of normal Bengalee subjects too, and the results with some (mostly University students of age 20-25 years) are represented in Table II.

TABLE I (a)

Period of treatment in months.

Patient No.		0	1	2	3	4	5	6	7	8
16	Blood sugar	140.4	126.8	...	116.3	103.0	124.6	100.0	...	...
	Inorganic phosphate	3.0	2.740	...	2.524	2.222	1.818	1.600	...	...
17	Blood sugar	293.0	255.8	...	298.0	265.0	238.6	218.5	225.0	233.6
	Inorganic phosphate	2.827	2.783	...	2.425	2.769	2.700	1.600	1.800	1.740
18	Blood sugar	242.0	224.0	...	202.3	125.0	...	98.00	96.00	...
	Inorganic phosphate	2.945	2.550	...	2.387	2.189	...	1.934	1.743	...
19	Blood sugar	256.0	...	202.0	202.0	127.6	182.0	183.0	191.0	...
	Inorganic phosphate	2.900	...	2.500	2.070	1.667	1.590	1.653	1.670	...
20	Blood sugar	189.3	98.0	...	123.0	105.0	105.0	...	...	...
	Inorganic phosphate	3.400	2.209	...	1.809	1.620	1.667	...	...	...
21	Blood sugar	223.0	222.0	225.0	190.0	168.0	...	...	...	...
	Inorganic phosphate	1.525	2.100	1.810	1.702	1.740	...	...	...	...
23	Blood sugar	180.0	162.0	156.0	151.0	...	...	...	...	...
	Inorganic phosphate	0.667	1.223	1.727	1.700	...	...	...	...	...
24	Blood sugar	147.0	97.00	70.0	75.00	...	...	...	...	...
	Inorganic phosphate	2.500	1.500	1.72	1.700	...	...	...	...	...

N.B. Both blood sugar and inorganic phosphate values have been expressed in mgms. per 100 cc. whole blood.

TABLE I (b)

Period of treatment in months.

Patient No.		1.0	1.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10	11
5	Blood sugar	...	268.0	...	...	270.0	264.0	...	165.0	113.0	...	180.4	148.0	139.0	...
	Inorganic phosphate	...	2.800	...	...	2.600	2.504	...	2.435	1.830	...	2.222	1.809	1.740	...
8	Blood sugar	212.0	...	235.0	...	...	...	217.0	...	210.0	...	221.0	...	...	...
	Inorganic phosphate	2.764	...	2.441	...	...	...	1.725	..	1.600	...	1.650	...	...	...
9	Blood sugar	173.0	176.1	...	165.0	...	...	121.5	...	115.6	...	...	...	121.0	100.0
	Inorganic phosphate	2.900	2.850	...	3.102	...	...	2.708	..	2.000	...	...	...	1.905	1.750
13	Blood sugar	218.1	...	...	...	216.9	...	165.0	...	161.1	...	160.0	...	155.0	116.0
	Inorganic phosphate	2.800	...	...	...	2.610	...	1.706	...	1.538	...	1.667	...	1.660	1.699

N.B. Both sugar and inorganic phosphate values have been expressed in mgms. per 100 cc. whole blood.

TABLE II

Inorganic Phosphate in the whole blood of normal individuals.

Name of the subject.	Inorganic phosphate (mgms. per 100 cc.)	Blood sugar (mgms. per 100 cc.)
A.S.	2.102	90.0
S.C.	2.082	88.0
C.P.	2.201	91.0
A.B.	2.090	91.0
M.C.	2.100	85.0
N.B.	2.301	95.0
K.S.	2.098	84.0
N.C.	2.100	78.0
S.D.	2.099	75.0
P.R.	2.098	90.0
Average value	2.100	87.3
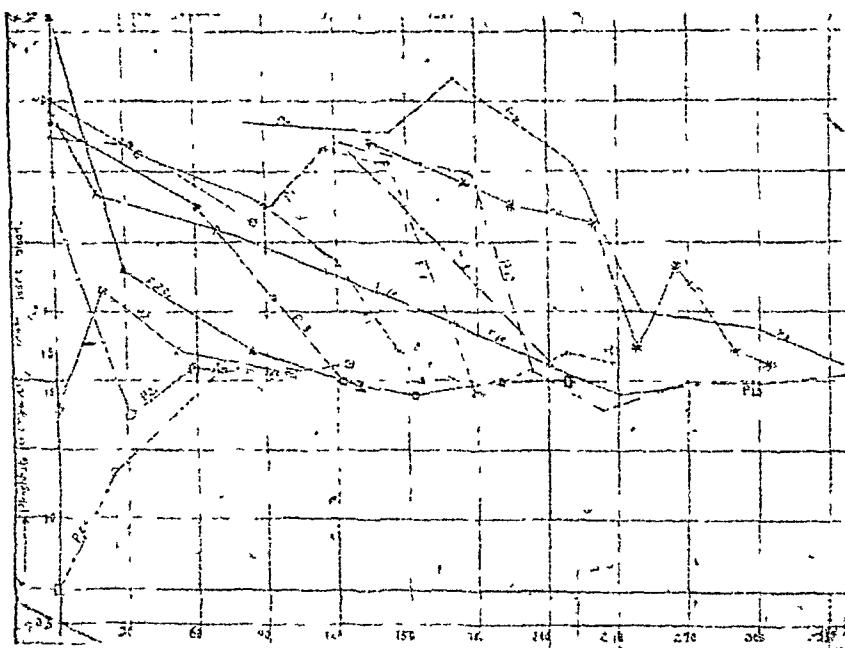
FIG. 1

Table III(*a*) and fig. 2 show the nature of the fall of inorganic phosphate in normal subjects with different quantities of glucose.

The nature of the fall of inorganic phosphate in diabetic subjects with oral administration of glucose (100 gms.) has been shown in Table III (*b*) and fig. 2.

TABLE III (a)

The effect of oral administration of glucose in different quantities on blood inorganic phosphate and blood sugar in the whole blood of normal healthy Bengalees.

Glucose in gms.	Blood inorganic phosphate (mgms. per 100 cc.)					Blood sugar (mgms. per 100 cc.)				
	Initial.	¾ hr.	1½ hr.	1¾ hr.	2½ hr.	Initial.	¾ hr.	1½ hr.	1¾ hr.	2½ hr.
100.0	2.100	1.760	1.500	1.800	2.000	95.0	168.0	101.0	95.0	93.5
150.0	2.100	1.850	1.500	1.820	2.000	95.0	168.5	103.6	95.4	91.0
200.0	2.100	1.820	1.500	1.800	1.990	95.0	167.4	103.5	95.5	91.0
Mean value	2.100	1.910	1.500	1.810	2.000	95.0	167.96	103.7	95.3	93.84

TABLE III (b)

The effect of oral administration of glucose (100 gms.) on blood inorganic phosphate and blood sugar in the whole blood of diabetic subjects.

Patient No.	Blood inorganic phosphate (mgms. per 100 cc.)									Blood sugar (mgms. per 100 cc.)								
	Initial.	½ hr.	1 hr.	1½ hr.	2 hr.	2½ hr.	3 hr.	3½ hr.	4 hr.	Initial.	½ hr.	1 hr.	1½ hr.	2 hr.	2½ hr.	3 hr.	3½ hr.	4 hr.
16	3.0	...	2.63	...	2.1	2.35	2.35	...	2.4	141.0	232.0	181.0	...	279.0	...	218.1	...	140.
24	2.5	...	1.9	1.79	1.75	...	1.95	...	...	147.0	...	188.0	160.0	150.0	...	148.1	...	...

FIG. 2

FIG. 3

Buel (12) believes that the blood cells contain practically no inorganic phosphate, almost whole of this occurring in the plasma. As the cells, according to the authorities (13) comprise of about 40% of the whole volume of blood, it is to be expected that the inorganic phosphate in the blood must be about 3/5 of that of the plasma.

If we take the maximum value of inorganic phosphate of diabetic individual to be 5 mgs. per 100 cc. plasma as recorded by others in most cases the calculated value for whole blood comes out to be 3 mgs. per 100 cc. While in the normal subjects taking 3.4 mgs. as the mean value for the same as found out by Bose and De (loc. cit) in majority of cases, the figure for the whole blood comes out to be in the neighbourhood of 2.1 mgms. per 100 cc. We too found this value (2.1 mg.) as the average figure for inorganic phosphate in the blood of normal healthy individuals. (Table II).

The lowest value for inorganic phosphates in the plasma after a test meal of 100 gms. of glucose to a normal subject, has been found by Bose and De to be 2.5 mgms. which corresponds to 1.5 mgms. in the whole blood. Since during active metabolism of carbohydrate there is a great demand for inorganic phosphate, we also contemplated to see if ingestion of varying amount of glucose in the same normal individual would cause difference in the degree of fall of inorganic phosphate in the blood or if there is some constant lower limit. It is interesting to note that the figures for inorganic phosphate value after giving a test meal of glucose of different quantities (100, 150, 200 gms.) even showed the same result and the lower limit (1.50 mgs./100 cc.) was found to be always constant.

DISCUSSION

It has been recorded by Nath, Chakraborty and Banerjee (11) that the excess of iron which is observed in the blood of severe diabetics, undergoes utilisation through amellin ingestion. As the inorganic phosphate also reaches the normal value in all cases finally, it was desirable to find the relationship, if any, between the inorganic phosphate and iron content of the blood in diabetics during amellin treatment.

Final values of inorganic phosphate of the diabetic patients and their corresponding values of iron have been plotted in fig. 3. The graph represents a straight line and thus indicates a very interesting phenomenon which can be stated as follows:—

"Though the values of iron in the blood of different diabetics are found to be distributed finally through some wide range (from 39.0 to 62.0 mgms. per 100 cc.), inorganic phosphate comes to the value which can be said to be more or less constant (1.6-1.75 mgms.) in all the cases, no matter whether the patient had abnormally high or low inorganic phosphate content in the blood initially."

Bose and De (loc. cit), observe that the value of inorganic phosphate in the plasma of a healthy normal individual goes on falling down within two hours' of injection of insulin (20 units) along with the fall of blood sugar, to the limit of 2.0 mgms. per 100 cc. plasma, which corresponds to 1.2 mgms. in the whole blood. This value had still a tendency to fall but no data being collected therein after two hours, nothing can be said about minimum value reached etc.

Unlike insulin amellin, however, does not cause the extra-demand of inorganic phosphate thus lowering its value far below the normal limit. It has rather been observed that the patients (P_{21} and P_{23}) who had very low figures at the beginning showed marked improvements within about 60 days of amellin ingestion, with rapid rise of phosphate. After a little fluctuation, the value was maintained more or less constant within the normal range.

Phosphate deficiency in case of diabetics was suggested by Gac (14) to be deficiency in food. But we are of opinion that this deficiency is not the result

of food insufficiency but may be due to some other factor which might have some influence in causing a drainage of phosphates from the system. This view can be supported from the fact that the urine of these two patients contained phosphate in abundance. Moreover, increase in the phosphate content of the blood to the normal range, while taking amellin, can not be accounted for by saying that food phosphate was increased during our treatment. We kept the diet of our patients more or less unchanged, rather the egg was cut off from the diet chart thus leaving no possibility of any increase in the food phosphate.

Another interesting point can be noticed regarding relation between the severity of the disease and the nature of the curve. Less severe is the case more stiff is the curve. Value with P_{20} comes down abruptly from 3.4 to 2.209 mgms. within 30 days and did never rise up again. His blood sugar was only 140.0 mgms. initially and that the case was a moderate one is clear from his tolerance curve as shown in fig. 2.

It can further be seen from Table I (a & b) that the lowest value of 1.5 mgms. for inorganic phosphate in the blood of diabetics, as observed after amellin ingestion, is not without significance. In case of normal subjects, ingestion of glucose, even in excess, can not lower the limit of inorganic phosphate value beyond such limit as 1.5 mgms. per 100 cc., however effective be the carbohydrate metabolism and phosphate demand within the system. But injection of insulin (20 units) has, however, the dangerous effect of not only causing hypoglycemia in normal individuals, but there is also a gradual fall of inorganic phosphate as pointed out previously.

In this way the superiority of amellin over insulin can also be established.

It has already been suggested (11) that there is possibly an "adjusting period" for an individual patient, length of the period varying according to the chronicity, duration etc. Here also we find confirmation of the same idea in this paper.

The adjusting period seems to be very high in the following cases where "slag curve" regarding the fall of inorganic phosphate was observed initially: —

P_9 is a Hindu widow aged about 50 years suffering from serious type of diabetes associated with severe heart troubles and excessive adiposity. She felt exhausted while trying to walk a little distance. She is now in normal state of health. It was only after 6 months that we observed a sudden fall in the inorganic phosphate in her case though relief in all symptoms was being reported since taking "amellin".

P_5 is an old man of 76 years old, suffering from diabetes for the last 25 years or so and bed-ridden for 10 months prior to our treatment. Rapid fall in the inorganic phosphate curve was observed only after 7 months and the patient can now take his morning walk alone.

P_{13} is an old man of 70 years who had 7.1% sugar in urine initially, the quantity of sugar eliminated in 24 hours being 285.2 gms. He became unconscious every now and then and all hopes of his life were gone. His "adjusting period" is found to be 7 months. Now he is almost in a perfect state of health.

Patient Nos. P_{20}, P_{19}, P_{24} which were moderate cases, showed, on the other hand, a rapid fall of inorganic phosphate from the beginning of the treatment thus indicating the presence of very small 'adjusting period' in these cases.

SUMMARY

(1) Effect of amellin in causing utilisation of inorganic phosphate in the blood of twelve diabetics, has been studied.

(2) This value, which was abnormally high in most of the cases, came down to the range of 1.6-1.75 mg. per 100 cc. of blood in all the cases, the normal value being 2.1 mg. as obtained from 10 healthy normal individuals.

(3) The lowest limit of inorganic phosphate in the blood of a normal subject after ingestion of glucose varying from 100 gr. to 200 gms., has been found to be 1.5 mg. and values in the neighbourhood of this, as observed in patients after taking amellin, have been suggested to be indicative of enhanced carbo-hydrate metabolism in the system.

(4) A relationship between the severity of the disease (diabetes) and the nature of fall in the value of inorganic phosphate has also been drawn ; less severe the case more rapid is its fall and *vice versa*.

(5) Initial phosphate deficiency in two cases, which rose up along with amellin ingestion, has been suggested to be due not to food insufficiency but due to some other factor which causes its extra drainage along with urine.

(6) Like insulin amellin does not cause fall of inorganic phosphate below 1.5 mg. and may thus claim its superiority over the latter in this respect too.

(7) Further confirmation regarding the conception of an initial adjustment period has been available.

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AVAILABLE IRON IN FISH. PART V. STUDIES ON THE MECHANISM OF ABSORPTION OF IRON IN THE IRON-COPPER-NUCLEOPROTEIN COMPLEX, OBTAINED FROM FISH-MUSCLE TISSUE

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The importance of iron in respiratory processes and in curing anæmias has long been recognised. But still there has been much controversy as to whether both ferrous and ferric iron or only the ferrous form is absorbed as such. Some are of the opinion that retention of iron in the body, blood formation and other aspects of iron metabolism are conditioned by the reduction of food iron in the alimentary tract and its absorption from the duodenum in the ferrous state [McGowan (1) ; Lintzel (2) ; Heubner (3) ; Starkenstein and Wedden (4) ; Reimann and Fritsch (5) ; Moore and others, (6) and Tompsett (7)]. The absorbed iron is believed to be oxidised in the blood and transported presumably as a ferric compound of globulin [Starkenstein and Harvalik (8)] to the liver where it is either utilized in hæmoglobin synthesis or stored largely in the reduced state. The above views regarding the reduction of food-iron in the alimentary tract and its ready absorption in the ferrous state are not based on any experimental evidence. From the considerations that, (*i*) in balance experiments, ferric iron has been found to be absorbed as easily as ferrous iron and (*ii*) in the treatment of anæmia, ferric iron produces as good a response as ferrous iron, the other school hold the view that ferric iron can be absorbed as such [Brock and Hunter (9) ; McCance and Widdowson (10) ; Whipple and Robscheit-Robbins (11)]. From the above considerations it cannot be definitely said that reduction of ferric iron to ferrous state does not take place prior to absorption from the alimentary tract.

In our previous publications (12-15), we have found that about 30 to 40% of the tissue iron is present in complex combination with nucleoprotein and copper and this iron can be quantitatively estimated by Hill's dipyridyl method (16). The iron of the complex, as obtained from fish tissue, is present in ferric condition and the complex possesses marked hæmoglobin regenerative capacity when fed to anæmic rats. It was obviously of interest to investigate whether reduction of this ferric iron at all takes places in the small intestine prior to absorption. The work to be described is concerned with this problem.

4

EXPERIMENTAL

Absorption is primarily a function of the small intestine where the conditions are very favourable both for digestion and for absorption, and the greater part of the available nutrients is supposed to be absorbed before the food mass reaches the ileocæcal valve. Various methods have hitherto been proposed and adopted by different workers to study the phenomenon of absorption. In studying the absorption of sugar (Cori, 17), animals were given sugar solutions by a stomach-tube. After a suitable time had elapsed, the animals were killed, the entire gastro-intestinal tract removed and total sugar remaining unabsorbed was determined. In order to study the influence of carbohydrates on the absorption of calcium and phosphorus, the method adopted by Bergeim (18) is as follows: To a standard diet is added a definite proportion of iron oxide and of the carbohydrate whose effect it is desired to study. The ratio of calcium and phosphorus to iron is determined for foods and fæces and the percentage absorption calculated. The above procedure is open to debatable conclusions, because it would be seriously misleading to consider the amount in the fæces as a measure of what had escaped utilization. It is known (Hawk, 19) that after utilization a large part of the phosphorus and a larger part of the calcium is likely to be eliminated through the intestine instead of through the kidney. This may happen with other elements too.

By establishing fistulas of the intestine the course of absorption in different parts of the tract has also been studied by some workers. Much of our information on absorption has also been obtained from the study of isolated intestinal loops (Hawk, 19) which retain their nerve and blood supply. Solutions can be injected into such loops and the contents removed at any time for analysis. The relative rates of absorption of various substances can thus be determined under controlled conditions.

But in our present investigation, as the intestine of the experimental animals was small in diameter, to obtain an isolated loop was not possible. Hence, the method has been modified as described below:

Healthy male rabbits of body weights varying from 1.2 to 2 kg. were taken. They were fasted overnight and anæsthetised on the day of experiment with urethane (1.6 g. per kg. of body weight). The abdomen was opened with all aseptic precautions, by a medline incision. A 4″ portion of the small intestine, about 2″ from the gastro-duodenal junction, was selected and two ligatures were tied at the two extremes of the selected loop. The experimental solutions were injected inside the lumen of the gut by a syringe with a fine needle. Iron solution, injected in each case, was 1 cc. containing 0.5 mg. of iron and in the case of blank experiment 1 cc. of normal saline was used. The pH of the test solution before injection was adjusted to 5.4. But in the case of I. C. N. complex, the pH was adjusted to 7.4, as the substance is insoluble in acid solution. The intestines were re-introduced into the abdominal cavity and the abdominal incision sutured up. The wound was covered by sterile dressings. After 3 hours, the abdomen was reopened, the loop of the intestine, experimented on, was isolated and the contents were washed with iron-free normal saline solution. The iron

was estimated in the washing by the method previously described. The results are given in Table I.

TABLE I

Body wts. of animals. (in g.)	Experimental solutions.	pH. of the test solution left after absorption.	Total iron left after absorption (mg.)	Original iron in the intestine (mg.)	Amount of iron absorbed (mg.)	Per cent. of iron absorbed (mg.)
1972	Ferric chloride	7.1	0.19	Nil	0.31	62
1760	,,	7.2	0.23	Negligible	0.27	51
1623	Ferrous ammon-sulphate.	7.2	0.21	.,	0.29	58
1571	,,	7.0	0.23	,,	0.27	54
1593	,,	7.1	0.28	Nil	0.22	44
1520	,,	6.9	0.27	,,	0.23	46
1310	Ferric chloride	6.9	0.23	Negligible	0.26	52
1349	,,	7.1	0.26	,,	0.21	48
1786	I. C. N. complex	7.1	0.18	,,	0.33	66
1660	,,	7.0	0.21	0.02	0.31	62
1783	,,	7.0	0.23	Negligible	0.27	54
1812	,,	6.8	0.21	,,	0.29	58

In order to investigate the possibility of simultaneous reduction and absorption of injected ferric iron through the intestinal membrane, the above experiment was repeated with ferric iron only. After 3 hours, the intestinal contents were washed as usual and made up to 20 cc. Aliquots were taken for the estimation of both the ferrous and ferric iron in each case left after absorption. The results are shown in Table II.

TABLE II

Body wts. of animals. (in g.)	Experimental solutions.	Total ferric iron left after absorption (mg.)	Total ferrous iron left after absorption (mg.)
1503	Ferric chloride	0.25	Nil
1498	,,	0.23	,,
1649	,,	0.26	,,
1853	,,	0.27	,,
1413	Ferrous ammon sulphate	Nil	0.27
1498	,,	,,	0.26
1802	,,	,,	0.22
1952	,,	,,	0.24
1548	I. C. N. complex	0.25	Nil
1602	,,	0.29	,,
1901	,,	0.23	,,
1957	,,	0.26	,,

Now, as we had observed earlier that ferric iron added to complex proteins, forms iron-protein complex, from which the iron cannot be recovered by washing with water or trichloroacetic acid extraction, it was considered desirable to see whether there is any chance of combination of the added ferric iron with the mucous membrane. In order to study this, healthy male rabbits, having approximately the same body weights as described before, were killed after anæsthetisation with urethane. The abdomen was opened and intestinal scrappings were taken in a 50 cc. conical flask with 10 cc. distilled water from a 4″: portion of the small intestine about 2″ from the gastro-duodenal junction. 0.5 cc. portions of ferric and ferrous iron were added separately to each of the flasks and incubated at 37° for 3 hours after which the mixture was filtered off, washed completely until free from soluble iron and the iron content of the filtrate and residue was determined. In order to estimate the iron content of the intestinal scrappings, blank experiments were also done without the addition of iron. The results are shown in Table III.

TABLE III

Body wts. of animals. (in g.)	Experimental solutions.	Quantity of iron added (mg.)	Iron in the filtrate and washings (mg.)	Original iron in 4″ portion of the intestinal scrappings (mg.)	Iron in the residue (mg.)	Iron combined with the scrappings (mg.)	Per cent. of iron combined (mg.)
1627	Ferric chloride	0.50	0.46	0.03	0.07	0.01	8
1532	,,	,,	0.45	0.02	0.07	0.05	10
1987	,,	,,	0.44	0.01	0.10	0.06	12
1930	,,	,,	0.46	0.03	0.07	0.01	8
1453	Ferrous ammon-sulphate	,,	0.50	0.02	0.02	Nil	Nil
1401	,,	,,	0.48	0.03	0.05	0.02	1
1827	,,	,,	0.50	0.03	0.03	Nil	Nil
1735	,,	,,	0.49	0.04	0.05	0.01	2

DISCUSSION

From Table I, it is found that within 3 hours about 44 to 66% of the injected iron is absorbed through the intestinal wall and there is practically no variations in the percentage of absorption whether the injected iron is in the ferric or ferrous form. The iron in the I. C. N. complex is also absorbed with equal rapidity even at an alkaline pH. Hence, these results indicate that absorption of iron from the small intestine does not depend on the acidity of the intestinal content as has been suggested by Tompsett (7). Tompsett has suggested further that as certain food-stuffs are capable of reducing ferric iron to the ferrous state at an acid medium *in vitro,* injected ferric iron might be reduced in the stomach which has a pronounced acid reaction and this reduction might be brought about by substances which are common constituents of diet. But our investigations apparently obviate this possibility, as the iron in the ferric state, after direct

injection in a selected part of the intenstine, where the pH is more or less neutral, has been found to be absorbed to an appreciable extent.

From the fact that ferric iron becomes non-dialysable *in vitro* in presence of phosphoproteins and phosphatides or complex proteins, Tompsett concluded that absorption of ferric iron is hindered in presence of any of these substances. But our results show the untenability of the above conclusion. Because, the iron present in the I. C. N. complex has been found to be equally absorbed through the intestinal wall as that of the injected ferric chloride even at pH 7.4, although at this pH, the iron of the same complex is non-dialysable *in vitro*, when allowed to be dialysed through parchment thimble and this complex iron, on being fed to anæmic rats, produces normal hæmoglobin regeneration. Absorption of food-constituents through the intestinal membrane is such a complex problem and is governed by so many factors that it does not seem to be explained by purely mechanical laws of diffusion as it takes place through a non-living membrane. There is evidence (Hawk, 19) that if the intestinal mucous membrane is killed or poisoned by sodium iodoacetate, it acts like an ordinary permeable membrane and the absorption mechanism is totally changed. So, by simple dialysis experiment with a non-living membrane *in vitro*, as has been done by Tompsett, it cannot be definitely ascertained whether a substance will be absorbed through the intestinal wall or not.

Now, the possibility of simultaneous reduction and absorption of injected ferric iron through the intestinal membrane cannot be overlooked. The results in Table II indicate that the iron, left after absorption in the intestine after 3 hours, is present in the same form in which it was injected. Had there been any possibility of an intermediate stage of reduction of ferric iron just prior to absorption then one could naturally expect some amount of reduced iron in the injected ferric iron, left in the intestine, if the intestinal contents are tested at any time. But in the present experiments, we have found no trace of reduced iron after injection of ferric iron.

It has been shown previously (13) that added ferric iron forms complex compounds with tissue materials and thereby cannot be recovered quantitatively by trichloroacetic acid extraction. There might be, therefore, some form of combination of the added ferric iron with the mucous membrane in our experiments, which would prevent extraction of the iron by simple washing with water and this might lead to the erroneous supposition that the disappearing iron has been absorbed. In order to test this possibility, experiments have been carried out to see how far the ferric and ferrous iron added to mucous scrappings *in vitro* can be subsequently recovered. The results are given in Table III. The results indicate that when iron in the form of ferric chloride is added to mucous scrappings about 8 to 12% of the added iron goes to form the insoluble complex and thus cannot be recovered by simple washing, but in the case of ferrous iron practically no combination occurs.

Summary

(1) Injected ferric and ferrous iron is absorbed with equal rapidity through the small intestine.

(2) Absorption of iron does not depend on the acidity of the intestinal content.

(3) The iron in the I.C.N. complex is absorbed to the same extent as that of the ferrous and ferric salts even at alkaline pH.

(4) Reduction of ferric iron is not necessary before absorption through the intestine.

(5) About 8 to 12% of the injected ferric iron forms insoluble complexes with mucous membrane and is not absorbed. Ferrous iron does not form such complexes.

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THE INFLUENCE OF DIET IN RESISTANCE TO INFECTION

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The importance of different factors in diet is being more and more appreciated. Differences in these factors have been held responsible for defective physical growths and diseased conditions have been associated with vitamin defeciencies. The diets of different races have a strict bearing with their physical characteristics apart from the other factors. The average western diet which is far more balanced than that in the East probably accounts for the better physical stamina of the people of the former countries. Whether the better diet has any influence on conferring on the body the power to resist infections is a question which has been asked by many.

The problem in its various aspects has been tackled by different workers. Most of the work has been with vitamins. We tried to see whether variations in protein content in diets influence immunity response. Work in this line is scanty. Watson (2 & 3) studied the influence of dried milk òn resistance and he showed that mice fed on dried milk were significantly more resistant to bacterial infections than those fed on cereals *e.g.,* oatmeal.

Orr and Gilks (1) made a survey of two African tribes. The Masai tribes living mainly on a diet of milk, meat and raw-blood were found to be not only physically surperior to the Kikuyu whose diet consisted mainly of rice and vegetables, but also appeared to be more resistant to certain types of infection. Whether the high rate of prevalence of different diseases in India has any

relationship to the defective diets can then be formulated which will not only have a nutritional value but perhaps also "anti-infective" value.

EXPERIMENTS

The experiments were performed on three different types of laboratory animals, guinea-pigs, rats and mice.

(A). GUINEA-PIGS.

24 Guinea-pigs were fed on casein, oats, butter-fat, dried yeast, shark liver oil and orange-juice of which a group of 12 (A) was given 22% protein and 55% carbohydrate and the other (B) 9% protein and 76% carbohydrate. Both groups were fed for six weeks. The average gain in weight of the individual animal in the protein group (A) was 80 grams and in the carbohydrate group (B) was 55 grams. The animals were then given intraperitoneally 500 million B ent. Gärtner. The mortality results were as follows, protein group 2 out of 12, carbohydrate group 10 out of 12. The animals were observed for 10 days.

The other batches of 12 guinea-pigs, fed on similar diet, were given weekly injections of killed emulsions of B. ent. Gärtner intraperitoneally for 4 weeks. One week after the last injection samples of blood were taken for agglutination and opsonic index. Blood was also taken from the animals on the usual laboratory diet for control. The results are given below.

TABLE I

Group.	Average agglutination titre.	Opsonic index.
Protein (A)	1 : 5120	1.1
Carbohydrate (B)	1 : 1280	0.6
Control	Negative	...

(B). RATS.

24 Rats were divided into two groups. They were fed on synthetic diet consisting of casein, corn starch, Osborne-Mendell salt mixture, butter-fat, yeast-tablets (Upjohn) and sharkliver oil. The diet of one group (A) consisted of protein (25%), carbohydrate (55%), fat (15%), salt-mixture (4%), with 0.35 grams of yeast tablet and shark liver oil.

The diet in the other (B) consisted of protein (9%), carbohydrate (71%), fat (15%), salt-mixture (4%) with 0.35 gram of yeast tablet and shark-liver oil. The average gain in weight of individual animal during the period in the protein group (A) was 75 grams and the carbohydrate group (B) was 40 grams. The animals were then given intraperitoneal injections of killed emulsions of B. typhi murium in doses of 50, 100, 250, 500, million organisms at four days' interval. The rats were bled one week after the last injection and the agglutination titre and the opsonic

index were studied. Control samples of blood were taken from animals fed on usual laboratory diet. The results are given below.

TABLE II

Group.	Average agglutination titre.	Opsonic index.
Protein (A)	1:160	1.4
Carbohydrate (B)	1:40	0.5
Control	Negative	...

(C). MICE.

24 Mice were taken in each group. One group (A) was given protein diet and the other (B) carbohydrate. The diet was same as of the rats in the previous experiments. They were fed for six weeks and the average gain in weight of the individual animal in protein group was 20 grams and in the carbohydrate group 12 grams. 12 Animals of each group were given intraperitoneal injection of 10 M.L.D. of B. typhi murium. The other two groups were given 10 M.L.D. of the culture by the mouth. The results are given below.

TABLE III

Group.	Number.	Method of injection.	Death.		After.		Hours.
Protein (A)	12	I.P.	2	5	3	2	...
Protein (A)	12	P.O.	0	1	4	5	2
Carbohydrate (B)	12	I.P.	7	4	1	...	...
Carbohydrate (B)	12	P.O.	2	6	4	...	...

DISCUSSION

The results of the experiments suggest that animals fed on a higher per cent of protein have developed a greater resistance to infection than those fed mainly on carbohydrate and minimum protein diet. It may be noted also that we have throughout our experiment used animal protein such as casein. The mortality rate in the guinea-pigs is especially appreciating. In both guinea-pigs and rats the agglutination test shows significant difference in titre among the (A) and (B) groups. Both the guinea-pigs and the rats, fed on protein diet, showed better opsonic indices than those fed on diet consisting mainly of carbohydrates. With the mice, the protein group (A) seems to show better resistance than carbohydrate group (B) if we take in consideration the days on which death takes place. We also found that death was expedited when cultures were injected intraperitoneally.

The experiments are, however, not conclusive. They only seem to indicate that a diet rich in proteins has a beneficial effect so far as resistance to infection in laboratory animals is concerned. We would like to point out also that proteins were of animal origin. Work is in progress to see whether vegetable proteins could also give the same results.

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SUMMARY

(1)   Different groups of animals were fed on diet rich in protein or carbo-hydrate.

(2)   The mortality rates after infection with virulent· organism were studied.

(3)   The serological tests to· measure immunity response were also undertaken.

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SOME OBSERVATIONS ON THE EFFECTS IN RATS OF ADDITION OF SPICES TO THE SOUTH INDIAN DIET

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Spices are popularly used almost all over India to add to the palatability of the dishes, and the hot stuffs, especially the dry chillies (*capsicum annum*) are too liberally used in certain parts of Bengal and all over the Madras Presidency where rice forms the staple diet of the people, which is deficient in its mineral content. A perusal of the composition of spices commonly used in India [Health Bulletin, (9)] shows that they are fairly rich in phosphorus and calcium content and to a certain extent in that of protein too. In order to ascertain if these accessory foodstuffs, that are so popularly used in India, have any supplementing effect upon the poor Indian diets (mainly based on rice) this work was taken up.

EXPERIMENTAL

The experiment was started with 24 young rats of average weight 55 gms., which were divided into two batches consisting of equal number of males and females. To the first group was given a poor south Indian diet, described in a recent paper by us [Pal and Singh, (7)] and to the second group was given the same diet in equal amount together with the following addition per rat per day:—

Dry chillies (*capsicum annum*)	...	0.32 gms.
Turmeric (*curcuma longa*) ...	...	0.32 gms.
Coriander seeds (*coriandrum sativum*)	...	0.16 gms.
Mustard (*Brassica Juncea*) ...	...	0.08 gms.

These were just proportional to adult human intake according to diet surveys of the common people of South India. The food was given in a cooked form as cooking has been shown to reduce the phosphorus content of the ordinary foods [McCarrison and Norris, (5) and also Ranganathan *et al*, (8)].

The animals were occasionally subjected to exposure to the sun throughout the experimental period lasting for eight weeks, during which their weight was recorded once every week. Table I shows the average weight of both the batches taken once a week for eight weeks.

TABLE I

Group	Initial weight.	1st week.	2nd week.	3rd week.	4th week.	5th week.	6th week.	7th week.	8th week.
1	55.2	53	54.7	57.2	61	65.5	66.5	71.5	74.7 g.
2	55.2	51.2	51	54.5	58.7	62.7	65.2	71.6	76.1 ,,
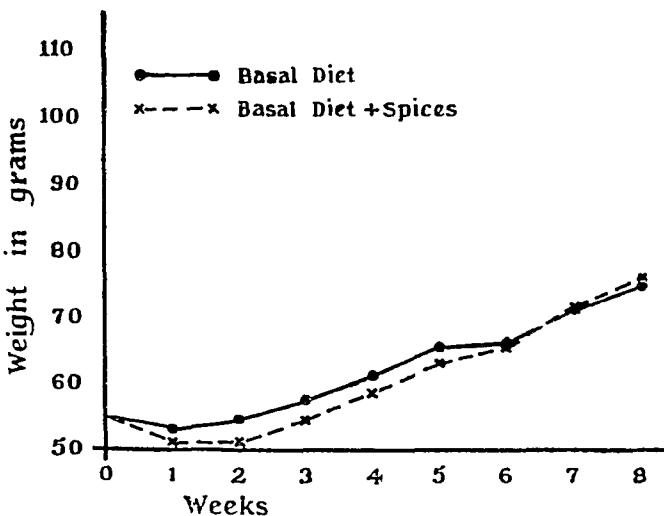
Six animals, three from each group, were kept in metabolism cages for six weeks, from the second to the seventh week, the first week being allowed for their readjustment to the changed condition inside the metabolism cages. The food was weighed in a dry condition everyday before it was prepared and given to the animals and the residual food and the excreta (fæces and urine) of each animal were collected carefully next morning for seven days, in order to note down the total quantity of calcium, phosphorus and nitrogen taken in and excreted during the whole week.

The calcium and phosphorus contents of the food and fæces were estimated by the standard methods. Those in urine were done by the method of Greenwald and Gross (3). The nitrogen of the food, fæces and urine was estimated by Kjeldahl's method.

Finally the animals were put under anæsthesia and their lengths were measured (from mouth to the end of the tail). Then blood was collected from the heart of three animals of each group together (as blood from one was not sufficient for the purpose) for estimation of serum calcium by the method of Kramer and Tisdall (4), that of serum phosphorus by Brigg's method (1) and of protein nitrogen by Macro-Kjeldahl method. According to Frisch *et al* (2) the non-protein nitrogen content of rat's blood is too small to be taken into account and hence this was not done.

One femur of each animal was dissected out and its length was measured. The final average length of the animals of both the groups was 25.5 cm. and that of femur of the first group was 2.23 cm. and that of the second group 2.33 cm.

The thyroid and parathyroid glands of the animals were also taken out for histological examination of the sections.

The following table shows the calcium, phosphorus and nitrogen content of the food taken, and excreta of both batches, estimated for six weeks.

TABLE II

Showing Intake, Excretion and Retention of Calcium (CaO), Phosphorus (P_2O_5) and Nitrogen (N) of the two groups of animals for six weeks.

Experimental week.	Group.	Quantity of CaO in food. g.	Quantity of CaO in excreta. g.	Total CaO retention g.	Total quantity of P_2O_5 in food g.	Total quantity of P_2O_5 in excreta g.	Total P_2O_5 retention g.	Total N in food g.	Total N in excreta g.	Total N retention g.
2nd week	I	0.181	0.035	0.146	0.630	0.323	0.307	2.764	0.888	1.876
	II	0.355	0.028	0.327	0.741	0.304	0.437	2.876	1.077	1.799
3rd week	I	0.161	0.047	0.114	0.494	0.361	0.133	2.186	1.247	0.939
	II	0.272	0.027	0.245	0.545	0.283	0.362	2.202	1.099	1.103
4th week	I	0.207	0.023	0.184	0.529	0.293	0.236	2.292	1.064	1.128
	II	0.349	0.028	0.321	0.741	0.284	0.457	2.515	0.946	1.569
5th week	I	0.185	0.036	0.149	0.551	0.358	0.193	2.285	1.574	0.711
	II	0.220	0.026	0.194	0.684	0.300	0.384	2.442	1.383	1.059
6th week	I	0.166	0.023	0.143	0.562	0.302	0.026	2.570	1.298	1.272
	II	0.242	0.023	0.219	0.635	0.262	0.373	2.486	1.177	1.309
7th week	I	0.174	0.058	0.116	0.590	0.429	0.161	2.672	1.393	1.279
	II	0.329	0.022	0.307	0.745	0.330	0.415	3.073	1.306	1.767

From Table II, the retention ratios of calcium and phosphorus were calculated, which showed a wide range of variations from 0.9 to 1.6 for both the batches. The calcium and phosphorus contents of the blood are recorded in Table III.

TABLE III

Group.	Calcium (mgs. per cent.)	Phosphorus (mgs. per cent.)
I	8.8	11.60
	8.4	12.25
	9.2	11.60
	8.8	10.90
Average.	8.8	11.58
II	12.4	9.66
	14.0	8.87
	12.8	9.58
	12.0	10.35
Average.	12.8	9.62

The protein content of the blood of the two groups did not show any variation, the average being 6.76 gms. per 100 cc. of blood for both the groups.

FIG. 1. Parathyroid gland, Group I showing fibrosis and hypoactive condition of the gland.
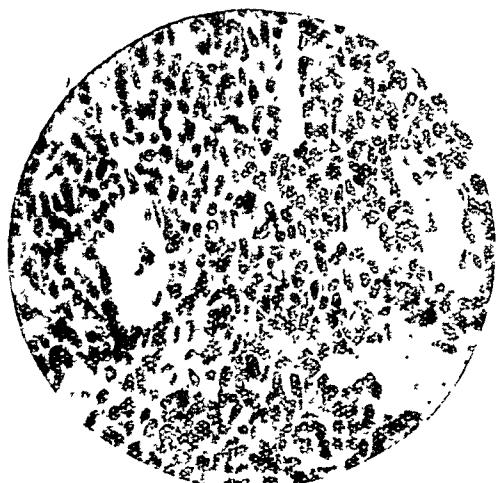
FIG. 2. Parathyroid gland, Group II showing normal structure.

Histological study of the parathyroid glands of group I showed big fibrous tissue bundles separating clusters of principal cells, with a very few blood sinuses in between. These glands were evidently in a hypofunctioning condition. The glands of the other group, however, showed much less quantity of fibrous tissues with well developed blood-sinuses—these showing more functional activity of the glands, than those of the first group. The thyriods did not reveal any

appreciable difference in the structure. Glands of both the groups showed thin alveolar epithelium with storage of colloid inside.

DISCUSSION

The above mentioned observations definitely show that spices which are rich in calcium, phosphorus and nitrogen content, in quantity that are usually used as condiments to add flavour and taste to the Indian food, cannot effectively supplement the mineral elements adequately to the poor Indian diet which is very much deficient in them. In this experiment the second group of animals that were on spiced diet fared no better than the controls ; neither the final weight nor the length, nor again the length of the femur of the former group showed any appreciable improvement over those of the latter. According to McClugage and Mendel (6) vegetables are much inferior to even salts of calcium as a source of calcium for the growing animals. This may be the reason why the animals did not improve on addition of spices as a source of calcium and phosphorus to their diet or it may be that quantitatively the mineral content added thus was too small to effect any material improvement in growth and nutrition. Addition of more spices to the diet is also not desirable as they are sure to act as irritant to the whole gastro-intestinal tract.

Some interesting factors were noticed in the composition of blood of the two groups of experimental animals. Group I showed some amount of hypo-calcæmia associated with higher phosphorus content while group II had calcium and phosphorus in normal proportions in their blood. There was, however, no difference in the protein content in the two groups of animals.

It is well known that the parathyroid gland has an intimate relation to the calcium and phosphorus metabolism. In this experiment the glands of the first group showed a hypofunctioning condition, characterised by increased amount of fibrous tissue and scanty blood supply, whereas the glands of the other group appeared to be normal in structure.

The structure of the parathyroid gland and the blood chemistry of the group I of this experiment were just the same as were found with the first group of animals in our previous experiment (Pal and Singh, *loc. cit.*). The condition of the second group was not altered in any way except addition of small quantities of spices to their daily diet. So the difference in the composition of blood as also the structure of the parathyroid glands of this group of animals must be due to the extra small quantities of calcium and phosphorus present in their diet, or in other words the small quantities of these mineral elements contained in the spices added, were responsible for keeping the calcium and phosphorus content of the blood and the structure of the parathyroid glands as well, normal.

The utilisation of nitrogen (and hence of protein too) was also slightly more for the second group of animals under the influence of the spices. This is prob-

ably due to the intake of the extra quantity of nitrogen of the spices that were added to the food.

CONCLUSION

Spices, though rich in calcium, phosphorus and nitrogen content, have no influence on nutrition and growth, in quantities usually used to increase the flavour and taste of the Indian dishes.

But even the small quantities of calcium and phosphorus, thus supplied to the poor Indian food (based on rice as a staple food), which is very much deficient in them, can offset the ill effects of mineral deficiency to a certain extent, by keeping the composition of blood so far as calcium and phosphorus are concerned, and also the structure of the parathyroid glands, normal.

My thanks are due to Dr. M. V. Radha Krishna Rao, for taking the photomicrographs.

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INVESTIGATIONS ON THE NEW ANTIDIABETIC PRINCIPLE (AMELLIN) OCCURRING IN NATURE. PART V. ITS INFLUENCE IN CAUSING RELIEF IN HYPER-CHOLESTEROLEMIA, AN INDEX TO TRUE PROGRESS IN CLINICAL DIABETES.

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It has long been recorded by several investigators (1, 2, 3) that there is a state that in case of hampered carbohydrate metabolism, the organism is forced general increase in the lipoid constituent of blood in clinical cases of diabetes mellitus. Removal of pancreas has been shown by Bloor (4) to cause hyperlipemia in experimental animals. Cambell (5) suggests that diabetic lipemia is an indication of greater demand for the metabolism of fat, because of the failure of supply of energy requirements from carbohydrate. Bodanskey and Bodanskey (6) state that in case of hampered carbohydrate metabolism, the organism is forced to take recourse to the metabolism of fat from the body reserves for the same.

That the production of diabetic lipemia is independent of the amount of food fat, within wide limits, has been shown by Newberg and Marsh (7), Marsh and Waller (8), Blatherwick (9) and others and it has been suggested by Rabinowitch (*loc. cit.*) that any treatment, which ameliorates diabetic symptoms and metabolic disturbances, diminishes lipemia. Man and Peters (10) observe that the rise and fall of blood cholesterol runs parallel with the change of total lipoids and hence the quantitative determination of steroids in the blood gives correct indication of the lipoid content at any particular time. Rabinowitch (11, 12) believes that the plasma cholesterol is the valuable index to the true progress of the diabetic patients and that the determination of blood cholesterol affords a better measure of the severity of diabetes than does the determination of blood sugar.

Amellin, which has recently been reported from this laboratory to bring about relief in hyper-glycemia associated with other complicated troubles (13, 14) and to cause a fall in inorganic phosphate (15), has been claimed to cause true progress in clinical diabetes and the present part will show its significance in relieving hyper-cholesterolemia.

It will also give some confirmation to the suggestion already made by us regarding metabolism of fat, caused in the diabetics, by this new anti-diabetic principle.

EXPERIMENTAL

Venous blood was drawn by means of a perfectly dry sterile syringe and collected in a tube containnig requisite amount of sodium citrate and was then mixed by rotating between the palms.

Cholesterol content of the whole blood was then determined by the method of Myers and Wardell (16). The technique, in short, is as follows:—

(i)   The measured quantity of blood is mixed with plaster of Paris and dried ;

(ii) the dried blood is extracted repeatedly with chloroform and the extract is treated with acetic anhydride and sulphuric acid ;

(iii) the estimation of cholesterol is made colorimetrically against a standard cholesterol solution, treated under identical conditions.

CHOLESTEROL CONTENT OF BLOOD IN NORMAL HEALTHY BENGALEES

The value of cholesterol present in plasma of normal Europeans and Americans has been found to vary from 160 to 230 mg. per 100 cc. while in case of Indians this value seems to be lower. Average values of 146 and 140 mg. per 100 cc. of whole blood have been recorded by Ghosh (17) and Bose and De (18) respectively, in cases of normal Indian subjects. A still lower average value (i.e. 116 mg. per 100 cc. whole blood) was observed by Boyd and Ray (19).

Moreover, the normal findings vary with the method employed. So before proceeding with the actual experiments on the diabetics, it was thought advisable to ascertain the normal value for the cholesterol content of whole blood of the normal Bengalees. For this, several normal blood samples were analysed for steroid content and the corresponding blood sugar values were also determined. The results are shown in Table I.

TABLE I

Name.	Age (years).	Cholesterol (mg./100 cc.)	Blood sugar (mg. 100 cc.)
A.S.	21	140.024	90.0
S.C.	22	138.580	88.0
C.P.	20	132.630	94.0
A.B.	23	125.980	91.0
M.C.	25	130.000	85.0
N.B.	24	134.450	95.0
K.S.	20	138.230	84.0
N.C.	24	120.300	78.0
S.D.	23	134.630	75.0
P.R.	27	134.700	90.0
Average	...	132.947	87.3

RELIEF IN HYPER-CHOLESTEROLEMIA

That amellin may claim to bring about proper metabolism of lipoid in diabetics has been indicated from a systematic study of blood-steroid at regular intervals.

It will be seen from Table II that even the values as high as 280 mg. per 100 cc. whole blood, came down to the normal range, which varies from 120 to 140 mg., within a few months' time ; the results are also graphically represented in fig. 1. It is interesting to note that the final values of blood cholesterol reach almost the same limit in all the patients, no matter whether their initial values for the same were very high or low. It can also be noticed (fig. 1) that in most cases the sterol value falls below the normal limit and soon rises and is maintained at a constant level.

Table II(a) shows the results with four patients, where estimation of sterol was not made within first four months of their treatment. This also gives similar nature of fall of cholesterol (fig. 2).

RELATIONSHIP BETWEEN HYPER-GLYCEMIA AND HYPER-CHOLESTEROLEMIA

Though it has not been possible to find out a definite relation between the amount of blood sugar and blood cholesterol in general, it will be evident from fig. 3 that the nature of the curve showing fall in blood-sugar, as a result of ingestion of amellin, is almost similar to that of corresponding values of inorganic phosphate and sterol as far as a particular individual case is concerned.

EFFECT OF GLUCOSE ON THE STEROL CONTENT OF THE BLOOD OF NORMAL AND DIABETIC CASES

It was thought desirable to see the effect of oral administration of glucose on the sterol content of blood in the normal as well as diabetic persons. For this, glucose was given to the patients or the normal subjects after 12 hrs'. fasting, and sugar and sterol estimated at some regular intervals. Results are shown in Table III.

TABLE II

Showing the relative lowering of blood sugar, cholesterol and inorganic phosphate in mg., caused by oral administration of amelin.

Patient No.		Period in months.											
		0	1	2	3	4	5	6	7	8	9	10	11
P. 16.	Blood sugar	110.4	126.8	...	116.3	103.0	124.6	100.0	100.0	...	...	...	...
	cholesterol	265.0	200.5	...	169.0	140.3	117.6	134.7	134.0	...	...	...	...
	Inorganic phosphate.	3.00	2.74	...	2.52	2.22	1.82	1.60	1.63	...	...	...	...
P. 17.	Blood sugar	293.0	255.8	...	298.0	265.0	238.6	248.5	225.0	233.6	150.6	...	...
	cholesterol	260.5	203.7	...	200.5	256.7	200.0	142.9	143.0	138.6	138.0	...	...
	Inorganic phosphate.	2.83	2.79	...	2.43	2.77	2.70	1.60	1.80	1.74	...	...	...
P. 18.	Blood sugar	242.0	224.0	...	202.3	125.0	...	98.0	96.0	...	...	...	...
	cholesterol	263.4	216.5	...	183.9	133.3	...	132.6	132.0	...	...	...	...
	Inorganic phosphate.	2.95	2.53	...	2.39	2.19	...	1.93	1.74	...	...	...	...
P. 19.	Blood sugar	256.0	...	201.0	202.0	127.6	182.0	183.0	191.0	...	145.4	...	141.6
	cholesterol	158.6	...	147.7	151.5	115.0	133.3	133.3	183.0	...	133.5	...	134.5
	Inorganic phosphate.	2.90	...	2.50	2.07	1.67	1.59	1.65	1.67	.	...	...	...
P. 20.	Blood sugar	189.3	98.0	...	123.0	105.0	105.0	...	100.0	...	99.0	...	...
	cholesterol	210.5	202.0	...	186.0	130.5	135.5	...	135.0	...	134.7	...	...
	Inorganic phosphate.	3.40	2.21	...	1.81	1.62	1.67	...	...	...	...	...	...
P. 21.	Blood sugar	223.0	222.0	225.0	190.0	168.0	...	...	...	130.0	...	...	...
	cholesterol	200.8	220.1	198.0	115.1	132.5	...	130.0	...	...	...	...	...
	Inorganic phosphate.	1.53	2.10	1.81	1.70	1.74	...	...	...	...	...	...	...
P. 23.	Blood sugar	180.0	162.0	156.0	151.0	...	...	...	...	...	...	...	...
	cholesterol	166.7	166.0	142.9	138.0	...	...	...	...	...	...	...	...
	Inorganic phosphate.	0.67	1.22	1.75	1.70	...	...	...	...	...	...	...	...
P. 24.	Blood sugar	147.0	97.0	70.0	75.0	...	...	...	...	...	...	...	...
	cholesterol	220.0	169.6	130.0	183.5	...	...	...	...	...	...	...	...
	Inorganic phosphate.	2.50	1.50	1.72	1.70	...	...	...	...	...	...	...	...

TABLE II (a)

Patient No.		Period in months.														
		4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	11.0	12.5
P. 5.	Blood sugar	...	268.0	...	...	270.0	264.0	...	165.0	143.0	...	180.4	148.0	139.0	...	105.0
	cholesterol	...	280.0	...	...	240.1	210.0	...	181.7	131.8	...	123.5	118.3	133.3	...	134.7
	Inorganic phosphate	...	2.80	...	...	2.60	2.54	...	2.44	1.83	...	2.22	1.81	1.74	...	1.70
P. 8.	Blood sugar	242.0	...	235.0	...	...	...	217.0	...	210.0	...	221.0	...	...	100.8	...
	cholesterol	270.3	...	200.0	...	...	...	166.7	...	137.0	...	133.3	...	...	132.6	...
	Inorganic phosphate	2.76	...	2.44	...	...	...	1.73	...	1.60	...	1.65	...	...	1.70	...
P. 9.	Blood sugar	173.0	176.4	...	165.0	...	...	121.5	...	115.6	...	...	...	121.0	116.0	132.6
	cholesterol	274.0	236.5	...	185.8	...	...	142.9	...	138.6	...	...	...	117.9	134.0	...
	Inorganic phosphate	2.90	2.85	...	3.10	...	...	2.71	...	2.00	...	...	...	1.91	1.70	...
P. 13.	Blood sugar	218.1	...	...	...	216.9	...	165.0	...	164.1	...	160.0	...	155.0	...	...
	cholesterol	200.0	...	...	...	215.6	...	195.5	...	180.5	...	160.0	...	134.5	...	...
	Inorganic phosphate	2.80	...	...	...	2.64	...	1.71	...	1.54	...	1.67	...	1.66	...	...

TABLE III
The effect of oral administration of glucose on the sterol content of blood (Normal and Diabetic subjects).

(a) Normal subjects.

Name.	Glucose in g.	Blood sugar mg./100 cc.					Blood cholesterol mg./100 cc.				
		Initial.	$\frac{3}{4}$ hr.	$1\frac{1}{4}$ hr.	$1\frac{3}{4}$ hr.	$2\frac{1}{4}$ hrs.	Initial.	$\frac{3}{4}$ hr.	$1\frac{1}{4}$ hr.	$1\frac{3}{4}$ hr.	$2\frac{1}{4}$ hrs.
N.C.	100	95.0	168.0	104.0	95.0	93.0	120.3	122.5	120.0	120.2	120.1
N.C.	200	95.0	167.4	103.5	95.5	94.0	120.3	128.0	120.3	120.1	120.4

(b) Diabetic subjects.

| Subject.	Glucose in g.	Blood sugar mg./100 cc.			Blood cholesterol mg./100 cc.		
		Initial.	1 hr.	2 hrs.	Initial.	1 hr.	2 hrs.
P. 32	150	316.0	415.0	442.0	Very low	62.5	107.2
P. 34	150	256.0	382.0	402.0	160.0	225.0	136.0
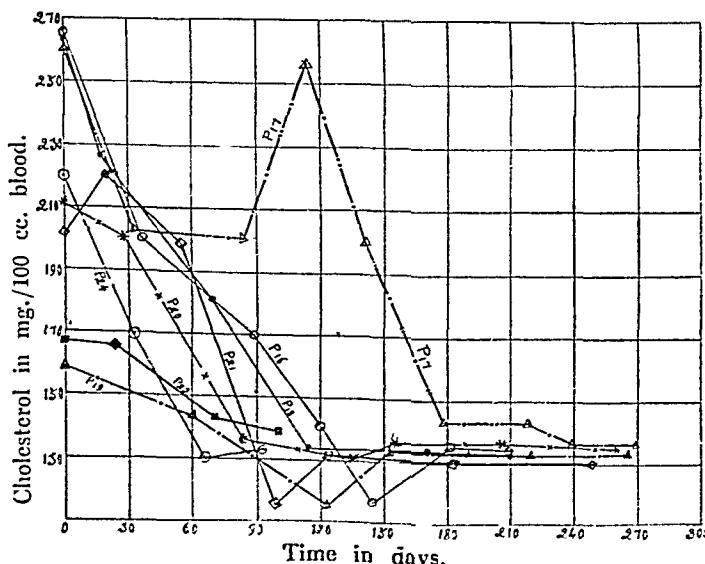
FIG. 1
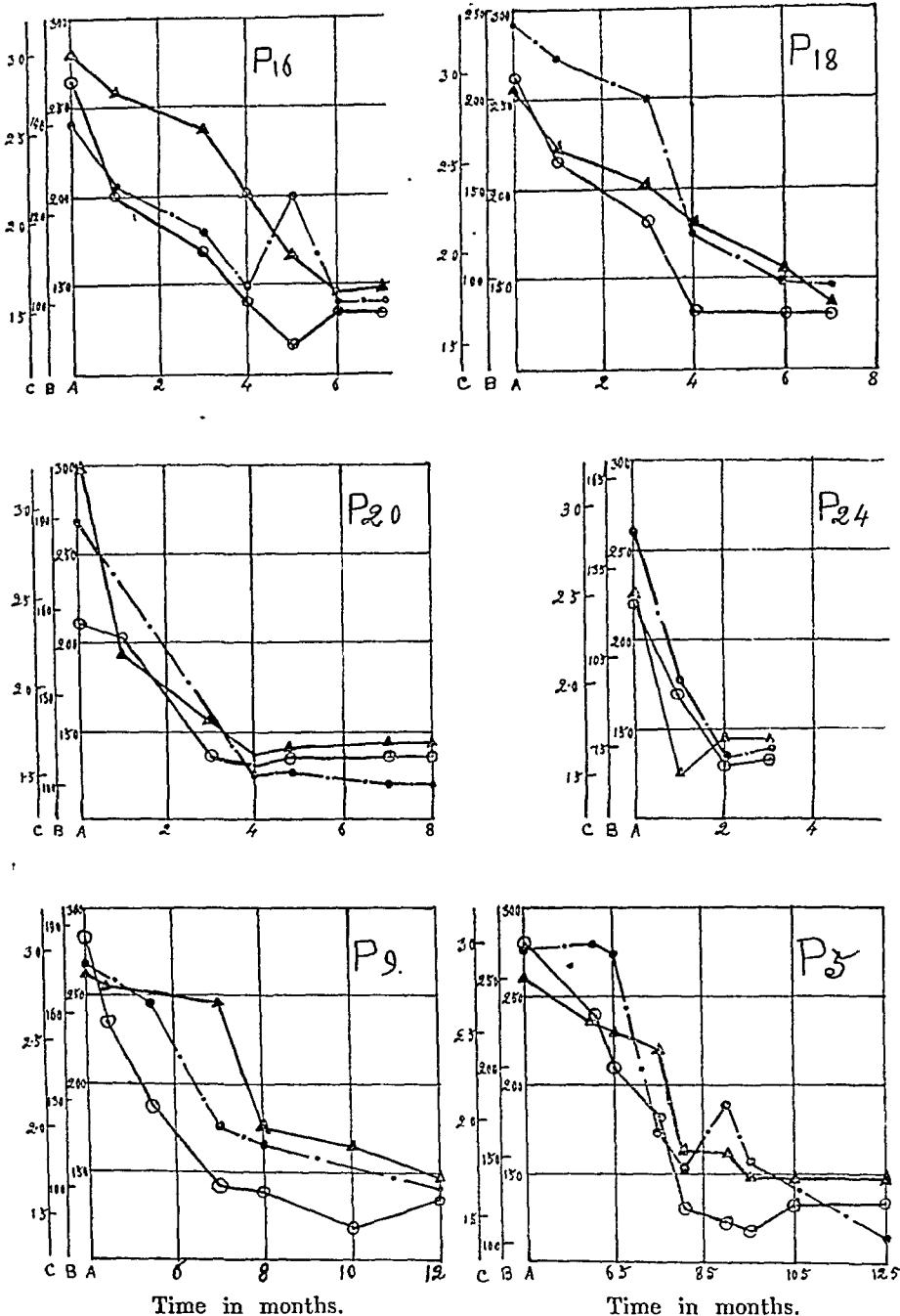
FIG. 3

Blood cholesterol—indicated by *Scale A* and represented by the curve

Blood sugar—indicated by *Scale B* and represented by the curve ●—●—

*Blood inorganic phosphate*—indicated by *Scale C* and represented by the curve △—△—△
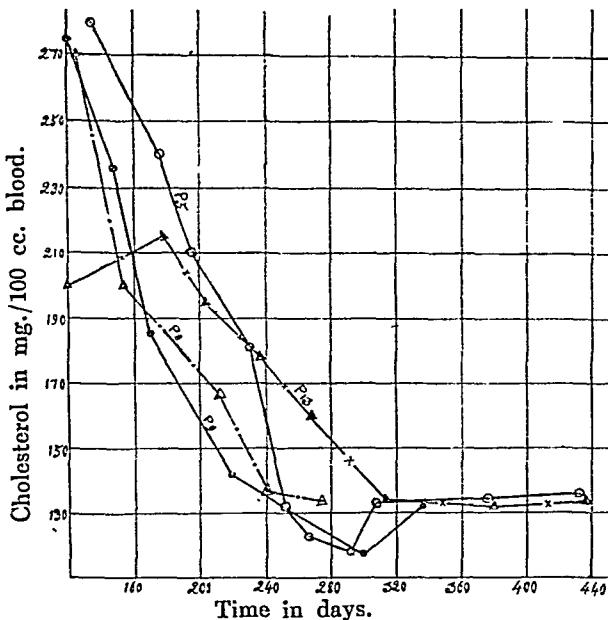
FIG. 2
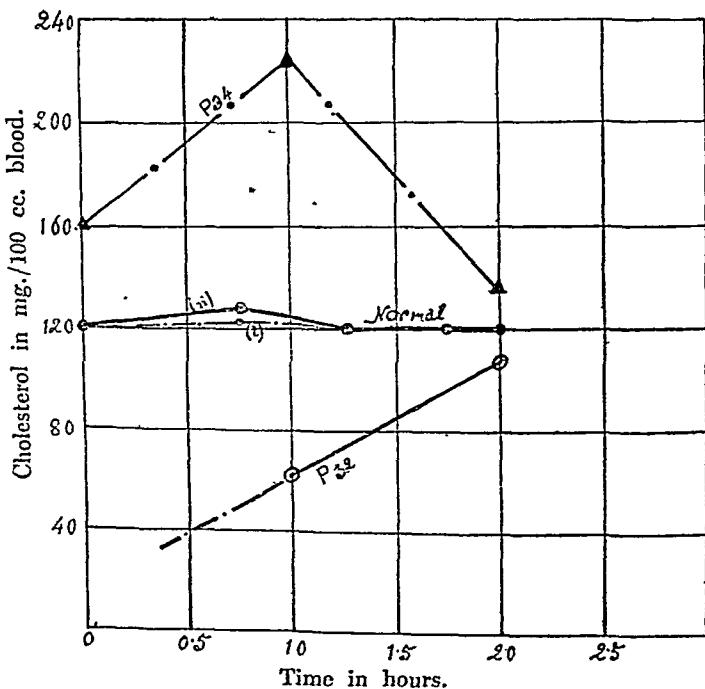
FIG. 4

DISCUSSION

It can be noticed from fig. 3 that reduction in hyperglycemia also runs parallel with that in hyper-cholesterolemia as far as an individual case is concerned. This indicates that increase in blood sterol and hence of blood fat may also be a physiological response to the greater demand for energy when the blood sugar is not in a position to meet it alone and gives a direct proof to the suggestion of Cambell (*loc. cit.*) that diabetic lipemia is an indication of greater demand for metabolism of fat. There is also another important point to notice. It can be seen from fig. 2 that the values of blood cholesterol, after taking amellin for 4 months, were found to be very high (280 mg.) in the severe cases suffering for a pretty long time *i.e.* more than 10 years. This may suggest the possibility of two things—either the initial values of cholesterol in these severe cases were far greater than the values observed after four months or the fall in those values might have begun very late, thus remaining constant for a long period. While comparing with sterol values of the moderate cases with those in the severe ones, it can be seen that the time required for the severe cases to reach the normal sterol limit is far greater than that in milder cases. This period varies from nine to ten months in these cases, while in milder ones normal value is reached within only two or three months' time. Similar fall in the inorganic phosphate content has also been observed in case of severe diabetics (15).

Though we observed similar fall in the amount of inorganic phosphate and cholesterol in blood of diabetics, after oral administration of amellin for several weeks, it can be noted with interest that quite different results are obtained for these two constituents after oral administration of glucose.

It has previously been recorded by us (15) that along with the rise in blood sugar, as a result of intake of glucose for sugar tolerance curve, there is at the beginning marked fall in the inorganic phosphate in case of normal subjects, and when there is a tendency of the blood sugar value falling downwards, there is again a rise in inorganic phosphate. This confirms very nicely the process of esterification of hexose with phosphate for its proper utilisation. The values for inorganic phosphate, in case of severe diabetics, under identical condition, seemed to be more or less constant for few hours, which signifies inability or loss of power of esterification in diabetics.

From the results of sterol estimation, after oral administration of glucose, it can be seen, however, that there is no such fall in case of normal beings; there is but very little rise in the value of steroid, even inspite of giving as large as 200 g. glucose orally and the original value is soon restored, for utilisation of sugar goes on regularly in a normal system and the requisite energy can be had mainly from it.

In case of diabetics, however, under identical condition (*i.e.* after giving glucose orally) there is marked rise in the blood steroid. But it is to be noticed from Table III and Fig. 4 that even in a serious case (p. 34) the maximum sterol value is reached within an hour, and within two hours a value less than the initial one is obtained. But the value for sugar goes on increasing even up to two hours of experiment. This shows how increase of the glucose concentration in

the blood sets an impetus to utilisation of sugar, thus lowering the demand for excess of lipoids in connection with giving body energy and thus gives support to the recent findings of Roy (20) regarding his new conception in the treatment of diabetes by injection of glucose.

The sterol value of P_{32}, a female case, greatly emaciated, (body wt. 95 lbs., age 30 years) was almost negligible during fasting condition. This, however, began rising on ingestion of glucose though its value did not reach the normal limit even in two hours' time.

SUMMARY

1. Effect of amellin on the steroid content of the blood of 12 diabetics has been studied.

2. With the progress in all the symptoms of the disease, there has been observed fall in sterol content (which is generally high in cases of diabetics) to the normal value.

3. The average normal value in young Bengalees has been found to be 133 mg./100 cc. blood.

4. The nature of the lowering of blood cholesterol is almost similar to that of inorganic phosphate as well as blood sugar.

5. Oral administration of even 200 g. of glucose to a normal subject causes an increase of only 8 mg. cholesterol after 45 minutes, and this value falls down soon to the original limit, while in case of diabetics the rise of blood sugar under the same condition accompanies the rise in cholesterol content of blood to a very great extent.

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VITAMIN C AND CARBOHYDRATE METABOLISM. PART I. THE EFFECT OF VITAMIN C ON THE GLUCOSE TOLERANCE TEST IN GUINEA-PIGS

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It has been reported that the administration of vitamin C is useful in the treatment of diabetes mellitus. Stepp, Schröder and Attenburger (1) showed that the administration of 300 mg. of vitamin C produced a fall in the blood sugar level of the diabetic and the normal subjects. A deficiency in the vitamin C-status of the body of the diabetics was observed by Pfleger and Scholl (2). They also observed that when vitamin C was administered in conjunction with insulin smaller doses of the latter were sufficient to check hyperglycæmia and glycosuria. Van Der Loo (3) described the improvement of two patients with moderate diabetes under treatment with vitamin C· in daily doses of 300 mg. Oshima, Terashima and Matsutani (4) showed that intravenous injection of 300 mg. of vitamin C in the diabetics diminished the blood sugar level and also the urinary excretion of sugar. That the administration of vitamin C improved the carbohydrate tolerance of the diabetics was observed by Dienst, Diemer and Scheer (5). Hosokawa and Sikinami (6) found a slight decrease in blood sugar and a more marked increase in blood glycogen in healthy individuals after the injection of vitamin C. According to the authors the action of vitamin C seemed to be similar to that of insulin. Hjorth (7) pointed out that in persons with a low serum ascorbic acid level the sugar tolerance curve definitely improved after the intravenous injection of massive doses of ascorbic acid. In seven out of eleven cases of diabetes, Turchetti and Schirosa (8) observed that the administration of vitamin C after a glucose tolerance test caused a smaller increase in the blood sugar level than the glucose tolerance test alone. In experiments with guinea-pigs Sigal and King (9) showed that tolerance to glucose is diminished during progressive stages of scurvy.

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Contrary to the above findings Izri (10) observed that the blood sugar of diabetic patients was not distinctly affected by vitamin C. Owens, Wright and Brown (11) made a survey of the vitamin C-nutrition of one hundred and twenty-five diabetic patients. These authors did not find any correlation between the vitamin C-status and the diabetic state of the patient. They also (12) did not find any significant difference in the insulin requirement of· sixteen diabetic patients, receiving a daily dose of 300 mg., 600 mg or 1,200 mg. of ascorbic acid for varying periods. In this connection it is of interest to study the utilisation of glucose under different conditions of vitamin C-metabolism.

The glucose tolerance test was performed under the following conditions: (a) in scorbutic guinea-pigs, (b) in normally fed guinea-pigs, (c) in guinea-pigs receiving normal diet and also injections of ascorbic acid, (d) in partially pancreatectomised guinea-pigs and (e) in partially pancreatectomised guinea-pigs receiving injections of heavy doses of vitamin C.

EXPERIMENTAL

Five groups of normal healthy guinea-pigs were taken. Each group contained 10 to 15 animals. The first group was kept on a scorbutic diet (13) for a period varying from 18 to 25 days. The second group of the animals received for four weeks normal diet consisting of green grass, germinated gram and vegetables. The third group of guinea-pigs, which received normal diet for two weeks, was given a daily injection of 100 mg. of vitamin C for three consecutive days. This dose of ascorbic acid was found by the author (14) to saturate the body of the animal. In the fourth group of guinea-pigs, which was placed on normal diet for two weeks, about half of the pancreas was removed from each animal by a small mid-line abdominal incision. The animals were anæsthetised with ether. Within fifteen minutes after the operation the animals moved almost like normal animals and took food. They passed urine and fæces in the same evening. On the second day of the operation the animals were placed separtely in metabolism cages and the urine was collected, toluene being used as a preservative. On the third day of the operation the glucose tolerance test was performed. In the last group, after partial pancreatectomy, the guinea-pigs received daily 100 mg. of ascorbic acid by injection for three consecutive days after the operation.

The animals of all the groups were fasted overnight before the glucose tolerance test was performed. The blood was taken from the heart and a 50% solution of glucose (0.2 g. per 100 g. of body weight) was administered per mouth. Blood samples were taken from the heart at intervals of 40 minutes upto 200 minutes, after the glucose was administered. The blood sugar was estimated according to the method of Hagedorn and Jensen (15).

RESULTS

The fasting blood sugar values of scorbutic guinea-pigs varied between 105 mg. and 185 mg. per 100 cc. of blood. The blood sugar level was steadily rising and in most cases was maximal at 120 minutes after the administration of glucose after which it came down but remained at a higher level than the fasting blood sugar value. The results are given in Table I.

TABLE I

Glucose tolerance test in guinea-pigs on scorbutic diet.

Animal No.	Weight of animal.	Days on scorbutic diet.	Before glucose administration.	Mg. of glucose per 100 cc. of blood. After glucose administration.				
				40 m.*	80 m.	120 m.	160 m.	200 m
1	230 g.	18	112	178	328	337	279	*died*
2	232	18	112	112	162	162	162	*died*
3	310	21	133	337	385	385	385	289
4	320	21	185	167	187	148	118	*died*
5	218	22	167	191	230	230	189	162
6	232	22	117	200	200	209	*died*	...
7	260	23	130	257	269	257	249	*died*
8	237	23	110	295	318	321	269	226
9	282	21	105	182	213	257	200	155
10	270	21	128	211	219	259	230	*died*
11	313	25	117	117	151	183	238	261
12	277	25	149	178	214	232	235	203
13	265	25	167	222	276	306	296	209

The fasting blood sugar values in the group of guinea-pigs receiving a normal diet varied between 83 mg. and 153 mg. per 100 cc. Thus unlike Sigal and King (9) who reported higher fasting blood sugar levels in scorbutic guinea-pigs, we find that the fasting blood sugar values in normal and scorbutic guinea-pigs do not seem to differ significantly. Out of 13 animals studied, in 5 cases the maximum blood sugar reading was obtained 40 minutes after the administration of glucose and in 7 cases at 80 minutes. In one animal, however, the maximum blood sugar reading was obtained at 120 minutes. At the end of 160 or 200 minutes the blood sugar values were definitely less than the corresponding values of scorbutic guinea-pigs. The results are given in Table II.

TABLE II

Glucose tolerance test in guinea-pigs receiving normal diets.

Animal No.	Weight of animal.	Before glucose administration.	Mg. of glucose per 100 cc. of blood. After glucose administration.				
			40 m.	80 m.	120 m.	160 m.	200 m.
1	385 g.	139	311	345	315	...	239
2	520	122	154	206	195	...	150
3	452	153	223	270	223	...	153
4	415	122	159	191	191	...	159
5	500	131	363	290	288	*died*	...
6	500	126	133	172	153	...	126
7	400	138	234	201	*died*	...	...
8	475	129	195	186	165	157	...
9	510	131	174	122	182	died	...
10	635	112	122	104	125	125	...
11	425	83	159	185	163	149	...
12	550	117	150	206	183	150	...
13	550	115	165	247	209	136	115

m* denotes minutes.

The fasting blood sugar values of normally fed guinea-pigs receiving extra vitamin C by injection varied between 117 mg. and 159 mg. These values did not differ significantly from those of the other two groups. Of the 15 animals studied in this group in 5 cases the blood sugar level was highest at 40 minutes after the administration of glucose, in 7 animals at 80 minutes after the glucose administration and in 2 cases at 120 minutes. The blood sugar values at the end of 160 minutes and 200 minutes were a little higher than the corresponding values obtained in the animals of the previous group receiving no supplement of vitamin C. The trend of the blood sugar tolerance curve was similar to that of the guinea-pigs on normal diets and the curve seems to be at a slightly higher level (Fig. 1). The results are given in Table III.

o————o Partially pancreatectomised guinea-pigs.
•————• Partially pancreatectomised guinea-pigs receiving injections of vitamin C.
•—··—··—··—• Scorbutic guinea-pigs.
•—··—··—··—o Normally fed guinea-pigs receiving injections of vitamin C.
•— — — — —o Normal guinea-pigs.

Minutes after ingestion of glucose.
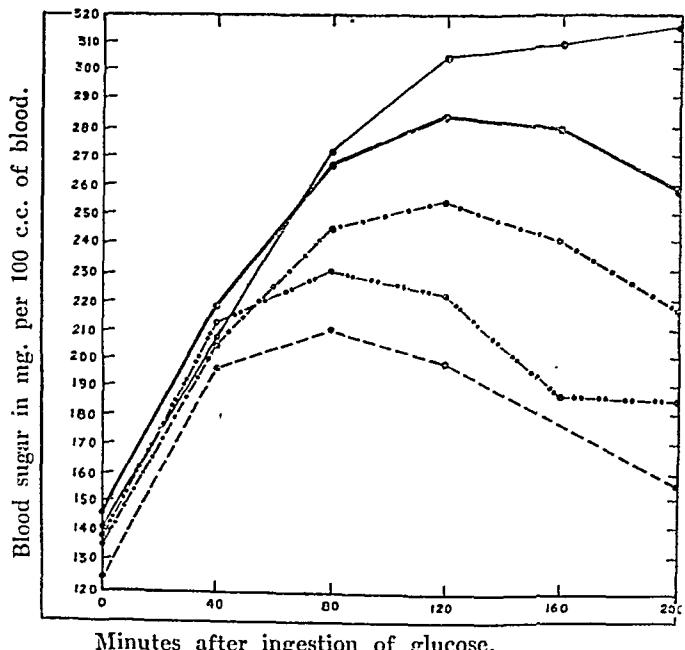
FIG. 1.

The partially pancreatectomised guinea-pigs passed traces of sugar in 24 hours' urine. The blood sugar values before the administration of glucose ranged between 93 mg. and 160 mg. per 100 cc. of blood and were practically the same as with the other group of guinea-pigs studied. The presence of traces of sugar in the urine and the low fasting blood sugar level definitely showed that the pancreatic tissue left after operation was just sufficient to control the fasting blood sugar. In all the 10 animals studied the blood sugar value was highest at 160 minutes after the administration of glucose and in 4 of them the high blood sugar level was maintained even up to 200 minutes after the administration of glucose. The results are tabulated in Table IV.

TABLE III

Glucose tolerance test in guinea-pigs on normal diets receiving injections of vitamin C.

Animal No.	Weight of animal.	Before glucose administration.	Mg. of glucose per 100 cc. of blood.				
			After glucose administration.				
			40 m.	80 m.	120 m.	160 m.	200 m.
1	370 g.	142	205	281	234	174	160
2	300	156	176	295	289	223	202
3	580	133	173	191	209	187	187
4	380	158	211	245	296	232	213
5	425	122	283	222	222	192	161
6	345	154	268	252	251	228	161
7	375	127	161	205	270	232	221
8	345	159	225	286	286	...	176
9	360	129	222	183	147	131	...
10	400	124	172	209	176	149	...
11	370	117	154	184	168	168	...
12	313	135	242	267	211	174	...
13	455	131	221	198	194	131	...
14	420	148	258	272	242	208	...
15	473	128	198	162	126	*died*	...

TABLE IV

Glucose tolerance test in pancreatectomised guinea-pigs.

Animal No.	Weight of animal.	Before glucose administration.	Mg. of glucose per 100 cc. of blood.				
			After glucose administration.				
			40 m.	80 m.	120 m.	160 m.	200 m.
1	275 g.	93	137	201	338	*died*	...
2	275	111	223	301	307	307	321
3	280	107	165	251	181	306	275
4	215	159	231	271	297	299	*died*
5	375	183	286	385	385	385	385
6	292	140	207	311	358	385	385
7	338	143	198	304	304	292	286
8	328	160	216	209	209	210	*died*
9	280	135	162	232	299	312	275
10	300	175	232	251	265	271	273

In the partially pancreatectomised guinea-pigs receiving vitamin C by injection the fasting blood sugar values varied between 107 and 131 mg. per 100 cc. blood and did not differ much from the corresponding values of the other groups.

Of the 14 animals studied in 4 cases the blood sugar value was highest at 80 minutes after the administration of glucose, in 7 animals the blood sugar value was highest at 120 minutes, in two animals at 160 minutes and in one the blood sugar value went on rising even after 200 minutes following glucose administration.   If these values are compared with the corresponding values of the partially pancreatectomised animals receiving no injection of vitamin C a definitely better tolerance to glucose is observed in most of the animals (Table V).

TABLE V

Glucose tolerance test in pancreatectomised guinea-pigs receiving injections of vitamin C.

Animal No.	Weight of animal.	Before glucose administration.	Mg. of glucose per 100 cc. of blood.				
			After glucose administration.				
			40 m.	80 m.	120 m.	160 m.	200 m.
1	375 g.	168	211	247	274	385	385
2	400	185	239	258	288	308	256
3	275	119	286	326	285	385	308
4	230	135	178	260	311	296	292
5	240	107	156	·228	214	199	199
6	260	140	237	249	256	260	268
7	272	128	196	296	284	224	220
8	250	117	207	302	249	239	160
9	300	135	163	169	187	187	*died*
10	232	149	233	286	290	290	250
11	165	167	196	260	247	222	214
12	540	169	220	272	337	310	249
13	450	171	280	284	315	...	306
14	460	140	252	299	342	338	*died*

The results were kindly statistically analysed by Dr. C. Chandrasekar of the All-India Institute of Hygiene and Public Health and the conclusions of the statistical analysis are as follows:

"The mean values of normal blood sugar in the five groups are not affected by the different treatments.   The initial rates of increase after feeding glucose are also of the same order in the five groups."

"The group of partially pancreatectomised guinea-pigs receiving injections of vitamin C have a small mean rate of increase in the blood sugar than the group of pancreatectomised animals which did not receive injections of vitamin C.   For the former group the time at which the blood sugar attains its maximum value is lower and so is the maximum value."

"There are indications that guinea-pigs on scorbutic diet have a higher mean rate of increase than those on normal diet and the time taken for them to attain the maximum value is also greater."

"The group of guinea-pigs who received injections of vitamin C in addition to normal diet behave very similarly to those on normal diet only."

DISCUSSION

The object of the present investigation is to study the effect of vitamin C in experimental diabetes with special reference to the utilisation of glucose. From the results it will be seen that scorbutic guinea-pigs do not utilise glucose like normal animals. This seems to show that vitamin C is necessary for normal utilisation of glucose. When extra vitamin C is injected into the normally fed guina-pigs, the blood sugar curve in the glucose tolerance test is not markedly altered. This indicates that the extra vitamin C is not required for the better utilisation of glucose. The normal utilisation of glucose is disturbed in partially pancreatectomised guinea-pigs. On the administration of vitamin C this disturbance is partly remedied as is evidenced from the fact that in most of the pancreatectomised animals the blood sugar curve in the glucose tolerance test comes down earlier as a result of the injection of vitamin C. The difference in the weights of the animals in the different groups studied is not expected to have any effect on the blood sugar values because, firstly, guinea-pigs cannot synthesise their vitamin C and secondly, the amount of glucose administered varied in proportion to the weight of the animal.

SUMMARY

1. The glucose tolerance test has been performed in scorbutic guinea-pigs, in normally fed guinea-pigs, in normally fed guinea-pigs also receiving vitamin C by injection, in normally fed partially pancreatectomised guinea-pigs and in partially pancreatectomised animals receiving vitamin C by injection.

2. Scorbutic guinea-pigs showed poor glucose tolerance when compared with guinea-pigs receiving normal diet alone or with the supplements of ascorbic acid. The blood sugar curve of the guinea-pig on normal diet compared fairly with the blood sugar curve of those animals which were receiving injections of vitamin C as well but the curve of the latter was at a little higher level. The glucose tolerance in the partially pancreatectomised animals with or without the supplement of extra vitamin C was very low as compared with the other groups of animals studied. Among the partially pancreatectomised animals, however, the animals which received vitamin C supplement showed a higher sugar tolerance than the animals receiving no injection of vitamin C.

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VITAMIN C AND CARBOHYDRATE METABOLISM. PART II. THE EFFECT OF VITAMIN C ON THE GLYCOGEN VALUE OF THE LIVER OF GUINEA-PIGS

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In diabetes mellitus, as is well known, there is a greatly diminished capacity for formation and storage of glycogen in the liver. Hirsh (1), Bartelheimer (2), Morelli and d'Ambrosio (3), Simizu (4) and Terada (5) showed that the administration of vitamin C produced an increase in the glycogen content of the liver of the experimental animals such as guinea-pigs or rabbits. It would, therefore, appear reasonable to invstigate the role of vitamin C in the deposition of the liver glycogen.

In the present investigation attempts have been made to study the glycogen content of the liver under the following conditions: (a) in scorbutic guinea-pigs, (b) in normally fed guinea-pigs, (c) in normally fed guinea-pigs receiving injections of vitamin C, (d) in normally fed partially pancreatectomised guinea-pigs and (e) in partially pancreatectomised guinea-pigs receiving heavy doses of vitamin C by injection. The relation of the glycogen content of the liver to its ascorbic acid content and to the fasting blood sugar level of the animals has also been investigated under the above-mentioned conditions.

EXPERIMENTAL

Five groups of normal healthy guinea-pigs were taken. Each group contained 13 to 22 animals. The first group was kept on a scorbutic diet for a period vary-

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ing from 21 to 23 days.   The second group of guinea-pigs was fed for two weeks on a normal diet as described before.   The third group of animals, fed on a normal diet for two weeks, was given a daily injection of 100 mg. of ascorbic acid for three days.   In the fourth group of guinea-pigs, which received normal diet for two weeks, the pancreas was partially removed as described before (6) and the animals were selected for the experiment on the third day after operation.   In the last group, the guinea-pigs after partial pancreatectomy received 100 mg. of ascorbic acid by injection for three consecutive days.

The animals in all the groups were starved overnight for 18 hours.   The blood was taken from the heart and the animals were stunned by a blow on the head.   The liver was removed.   The glycogen in the liver was precipitated by the method of Grattan and Jensen (7) and the glucose in the hydrolysed precipitate was estimated by the method of Hagedorn and Jensen (8).   The ascorbic acid in the liver was extracted by the method of Bessey (9).   The extract was titrated against a solution of 2: 6-dichlorophenol-indophenol, standardised according to the method of Menaker and Guerrant (10).   The blood sugar was determined by the method of Hagedorn and Jensen (8).   Twenty-four hours prior to killing, the animals of all the above groups were kept in individual metabolism cages and their urine was collected, toluene being used as a preservative.   The urine was tested for the presence of sugar with Benedict's reagent.   The results are shown in Tables I-V.

TABLE I
Scorbutic guinea-pigs.

Animal No.	Weight of animal (g.)	Days on scorbutic diet.	Blood sugar (mg. %)	Liver glycogen (mg. %)	Liver ascorbic acid (mg. %)	Sugar in urine.
1.	377	21	76	40	4.2	Present
2.	524	21	97	44	2.8	,,
3.	305	21	120	41	2.4	,,
4.	382	21	108	32	2.6	,,
5.	238	21	131	30	2.6	,,
6.	316	21	131	60	1.8	,,
7.	310	22	79	46	2.4	,,
8.	251	22	88	35	2.6	,,
9.	429	22	110	80	2.6	,,
10.	286	22	88	51	2.0	,,
11.	380	22	103	52	2.2	,,
12.	427	22	124	20	2.2	,,
13.	383	23	119	80	2.6	,,
11.	297	23	103	49	2.8	,,
15.	275	25	...	88	...	,,
16.	221	25	...	55	...	,,
17.	329	25	...	...	...	,,
18.	200	25	...	...	...	,,
Mean			...	97	...	,,
Standard deviation			105	50	2.6	
			17.6	23.8	0.27	

TABLE II

Normal guinea-pigs.

Animal No.	Weight of animal (g.)	Blood sugar (mg. %)	Liver glycogen (mg. %)	Liver ascorbic acid (mg. %)	Sugar in urine.
1.	470	83	540	5.0	Absent
2.	590	74	1360	6.0	,,
3.	180	83	1360	11.6	,,
4.	262	124	3850	13.2	,,
5.	338	113	1480	18.2	,,
6.	642	118	1460	15.2	,,
7.	605	113	4580	21.0	,,
8.	642	108	2530	21.0	,,
9.	385	139	2260	13.2	,,
10.	520	122	1840	13.2	,.
11.	452	153	2929	7.6	,,
12.	415	122	3442	12.8	,,
13.	500	144	910	9.4	,,
14.	500	126	2308	12.8	,,
15.	400	138	2214	20.8	,,
16.	375	110	1720	19.6	,,
17.	191	131	1900	29.0	,,
18.	240	134	2545	31.6	,,
19.	252	101	2327	29.0	,,
20.	280	120	3015	27.4	,,
21.	283	83	1985	27.3	,,
22.	188	120	2727	29.0	,,
Mean	...	116	2240	18.8	
Standard deviation		20.6	930.6	5.1	

TABLE III

Normally fed guinea-pigs receiving injections of ascorbic acid.

Animal No.	Weight of animal (g.)	Blood sugar (mg. %)	Liver glycogen (mg. %)	Liver ascorbic acid (mg. %)	Sugar in urine.
1.	205	131	1000	32.0	Absent
2.	473	128	4620	34.8	,,
3.	345	159	1864	44.4	,,
4.	375	127	1500	26.6	,,
5.	363	135	1842	30.8	,,
6.	380	158	936	17.8	,,
7.	425	122	1221	29.6	,,
8.	400	124	753	32.0	,,
9.	370	131	3729	36.2	,,
Mean	...	135	1941	31.5	
Standard deviation	...	13.19	1260.7	6.95	

TABLE IV

Normally fed partially pancreatectomised guinea-pigs.

Animal No.	Weight of animal (g.)	Blood sugar (mg. %)	Liver glycogen (mg. %)	Liver ascorbic acid (mg. %)	Sugar in urine.
1.	310	136	0	5.2	Present
2.	332	125	15	3.8	,,
3.	249	101	145	3.6	,,
4.	415	136	106	5.4	,,
5.	318	186	124	5.8	,,
6.	340	117	80	5.6	,,
7.	299	102	108	7.8	,,
8.	530	96	57	6.8	,,
9.	496	111	24	2.6	,,
10.	486	113	32	2.8	,,
11.	467	97	51	4.0	,,
12.	235	95	53	7.4	,,
13.	390	84	47	6.0	,,
Mean	...	115	75	5.1	
Standard deviation	...	25.6	21.0	1.6	

TABLE V

Pancreatectomised guinea-pigs receiving injections of vitamin C.

Animal No.	Weight of animal (g.)	Blood sugar (mg. %)	Liver glycogen (mg. %)	Liver ascorbic acid (mg. %)	Sugar in urine.
1.	410	165	100	28.6	Present
2.	345	147	195	20.0	,,
3.	447	111	125	28.6	,,
4.	519	108	250	26.4	,,
5.	600	110	260	21.4	,,
6.	445	120	215	41.4	,,
7.	170	111	190	14.2	,,
8.	355	143	285	14.2	,,
9.	227	110	479	20.6	,,
10.	209	119	241	40.0	,,
11.	233	110	895	35.6	,,
12.	290	124	1253	40.0	,,
13.	278	115	2710	33.8	,,
Mean	...	123	561	28.1	
Standard deviation	...	17.7	694.1	9.3	

DISCUSSION

The glycogen content of the liver is greatly diminished in scorbutic guinea-pigs. The glycogen content of the liver of normally fed guinea-pigs is not significantly changed by the injection of extra vitamin C. This indicates that extra vitamin C is not required for the better deposition of liver glycogen if there is sufficient vitamin C in the diet. In partially pancreatectomised guine-pigs receiving a normal diet but no extra vitamin C by injection, it is observed that the glycogen value of the liver is more or less comparable with that of scorbutic guinea-pigs. When the normally fed partially pancreatectomised guinea-pigs are given additional vitamin C by injection, better deposition of liver glycogen takes place. The vitamin C content of the liver is greatly diminished in both the scorbutic and the partially pancreatectomised guinea-pigs receiving no injection of vitamin C. In the partially pancreatectomised guinea-pigs which receive injections of vitamin C the ascorbic acid content of the liver increased like that of normal guinea-pigs given ascorbic acid by injection ; the corresponding glycogen values of these two groups of animals, however, are not comparable. The fasting blood sugar values of the different groups studied do not differ significantly. Both the scorbutic and the partially pancreatectomised animals excreted sugar in traces in their urine. This fact seems to indicate that the carbohydrate metabolism is similarly disturbed in both these conditions.

SUMMARY

1. The glycogen and the ascorbic acid values of the liver have been determined in scorbutic guinea-pigs, in normal guinea-pigs, in normal guinea-pigs receiving injections of vitamin C, in normally fed partially pancreatectomised guinea-pigs and in normally fed partially pancreatectomised guinea-pigs and also receiving extra vitamin C by injection.

2. The glycogen values of the liver are strikingly diminished in scorbutic and in partially pancreatectomised guinea-pigs. When the partially pancreatectomised guinea-pigs are given vitamin C by injection greater deposition of liver glycogen takes place. Considering the average glycogen value of the livers of normal guinea-pigs to be 100, the corresponding values in scorbutic guinea-pigs are 2.2, in partially pancreatectomised guinea-pigs, 3.7 and in partially pancreatectomised guinea-pigs receiving injections of vitamin C, 25.1.

3. The vitamin C values of the liver are greatly diminished in both the scorbutic and the partially pancreatectomised guinea-pigs receiving no injection of ascorbic acid.

4. Both the scorbutic and the partially pancreatectomised guinea-pigs show the presence of sugar in their urine.

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VITAMIN C AND ·CARBOHYDRATE METABOLISM. PART III. THE EFFECT OF VITAMIN C ON THE CHLORIDE CONTENT OF THE BLOOD OF GUINEA-PIGS

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Recent researches show that sodium chloride helps in the better utilisation of glucose in certain animals and it further helps the liver to store glycogen. Orten and Devlin (1) observed that when partially pancreatectomised diabetic rats and intact rats with a low glucose tolerance were given injections of sodium chloride along with glucose the tolerance to glucose was found normal. Crabtree and Longwell (2) reported that the administration of sodium chloride to rats increased the deposition of glycogen in the liver. It was further observed by McQuarrie, Thompson and Anderson (3) that in some human diabetics the injection of sodium chloride improved the utilisation of carbohydrate as manifested by decreased glycosuria, lowered fasting blood sugar level and decreased ketosis. It is therefore possible that the lowered glucose tolerance (4) and the diminished glycogen content of the liver (5) in scorbutic and experimentally diabetic guinea-pigs may be due to altered chloride metabolism in these conditions. The chloride content of the blood of normal and scorbutic guinea-pigs has, therefore, been studied.

EXPERIMENTAL

Two groups of normal healthy guinea-pigs were taken. The first group containing 19 animals was fed on a scorbutic diet for a period varying from 23 to 28 days. The second group, which contained 20 guinea-pigs, was placed on a normal diet for two weeks. The animals were starved overnight and the blood samples were taken from the heart. The chloride content of the blood was determined by the method of Whitehorn (6). The results are given in Table I, and the statistical analyses are given in Table II.

DISCUSSION

The blood chloride values in scorbutic guinea-pigs varied between 417 and 565 mg. per 100 cc. of blood with an average value of 494.6 ± 14.9 mg.. In normal guinea-pigs the blood chloride values ranged from 462 to 533 mg. with an average value of 494.4 ± 6.8 per 100 cc. of blood. We find that there is no statistically significant alteration in the blood chloride values in these two conditions. The disturbed carbohydrate metabolism produced by deficiency of vitamin C does not, therefore, seem to be due to any defect in the metabolism of chlorides.

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TABLE I
BLOOD CHLORIDE OF GUINEA-PIGS.

Scorbutic guinea-pigs.   *Normal guinea-pigs.*

Days on scorbutic diet.	Weight of animal.	Blood chloride (mg. per 100 cc.)	Weight of animal.	Blood chloride (mg. per 100 cc.)
23	193 g.	565	257 g.	497
24	209	550	273	494
24	431	493	242	496
24	296	565	210	491
24	348	490	290	492
25	250	417	186	489
25	303	485	177	529
25	364	488	176	493
25	406	488	213	494
25	311	473	180	494
25	276	484	197	490
26	312	487	166	488
26	297	488	221	462
26	250	480	272	492
26	295	489	256	493
28	304	484	232	485
29	282	487	225	490
29	190	490	222	475
29	248	496	222	511
...	...	...	240	533
Average	...	494.6	...	494.4
Standard deviation	...	32.58	...	15.208

TABLE II
Statistical analysis:

Standard error of difference	0.2
Difference of mean rates	8.23
t	0.024
Remarks	not significant.

SUMMARY

1. The blood chloride was determined in both the normal and the scorbutic guinea-pigs.

2. In the scorbutic guinea-pigs the blood chloride values varied between 417 and 565 mg. per 100 cc. of blood. In normal animals the blood chloride values varied between 462 and 533 mg. per 100 cc. of blood.

3. The change in the metabolism of carbohydrate observed in scurvy is not due to any defect in the chloride metabolism.

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OBSERVATIONS ON ALCOHOL AND OTHER DICHROMATE REDUCING
SUBSTANCES IN NORMAL BLOOD

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Accurate estimation of alcohol in the blood has been of inestimable value
in medicolegal work for determining the amount of alcohol consumed by an
individual. It does not, however, always indicate the degree of intoxication pro-
duced thereby. To assess the alcoholic intoxication or drunkenness of a person
it is sometimes necessary to combine the laboratory finding with a careful and
exhaustive clinical examination. The positive laboratory finding in most of these
cases as in all laboratory tests for diagnosis, does not carry one further than
helping him to give the opinion that the person consumed alcohol and was prob-
ably under its influence. The negative finding is, on the other hand, of immense
value in giving a definite opinion that the person charged of drunkenness was
not drunk at the time.

Enormous amount of work has been done, mostly by German workers, in
this direction and as usual a, considerable difference of opinion appears to exist
among them regarding the relation between the concentration of alcohol in the

blood and the degree of intoxication it produces. The general concensus of opinion is, however, in favour of fixing the minimum limit at 80 mg. per 100 cc, of blood [Smith & Glaister, (3)], at which concentration no intoxication has been known to take place.

If the Traffic Acts now enforced in the United Kingdom and other European countries are also introduced into this country, and they are likely to be introduced in near future, similar investigations for correct diagnosis by laboratory methods of drunkenness in a driver of a motor vehicles will be a necessity.

The usual method for determination of alcohol in the blood is to volatilise the alcohol by heat and to oxidise the vapour by a known amount of standard $K_2Cr_2O_7$ solution and H_2SO_4 to acetic acid (Southgate, 1936). The oxidation of alcohol is accompanied by reduction of a corresponding amount of $K_2Cr_2O_7$ to chromium sulphate. The unreduced portion of $K_2Cr_2O_7$ is then estimated by the usual iodometric method and the amount of alcohol is calculated from the amount of dichromate thus reduced. One c.c. of $N/10$ $K_2Cr_2O_7$ contains 4.9 mg. of $K_2Cr_2O_7$ and is equivalent to 1.15 mg. of alcohol as indicated by the following equation : —

$$2K_2Cr_2O_7 + 8H_2SO_4 + 3C_2H_5OH = 2Cr_2(SO_4)_3' + 2K_2SO_4 + 11H_2O + 3CH_3COOH.$$
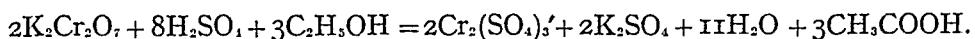
The reduction of dichromate is the essential feature of this method and the chemical reactions involved in the process indicate that any volatile and oxidisable substance present in the blood may also be volatilised along with ethyl alcohol and oxidised by $K_2Cr_2O_7$. The result obtained by this method does not, therefore, give the quantity of alcohol only but of a mixture of alcohol and other volatile reducing substances, such as ketone bodies and possibly some other volatile metabolic products and also some drugs. The alcohol content of normal blood being usually about 4 mg. [Sollmann, (4)] and the amount of ketone bodies being in the neighbourhood of 1 mg. per 100 grams of blood, the dichromate reduction figure in a healthy person not taking any alcohol, that is, the 'blank' or the 'normal value' should not, therefore, exceed 5 mg. which is the total of the above two figures. Actually, however, it is sometimes considerably higher than the above total in many healthy cases. Different workers have given different 'normal value'. In a series of cases the maximum was 20 mg. and the average was 3.7 mg. per 100 cc. of blood (Smith & Glaister, loc. cit.).

As the normal value should always be deducted from the alcohol content of the blood of the individual under examination, it is necessary to form a correct idea of this value in the case of Indians, because the figures worked out by the German and other European workers may not be applicable to them whose diets and modes of living are quite different from those of the people of Western countries. With this end in view, the blood of 50 healthy persons, men and women, was examined and the dichromate reducing figure or the 'normal value' was thus found out. The result has been recorded in the following tables.

TABLE I

Healthy people not addicted or exposed to alcohol in any way.
Figures indicate mg. of alcohol and other dichromate reducing
substance (expressed as alcohol) in 100 cc. of fresh blood.

Mean = 2.45.

Serial No.	Name.	Age, sex &c.		Occupation.	Result.	Remarks.
1.	K.N.B.	H.M.,	55	Chemist	Trace	After 4 hours' work in the alcohol laboratory it went up to 3.45.
2.	A.K.	M.M.,	32	,,	Trace	
3.	H.D.G.	H.M.,	42	,,	1.15	
4.	C.R.T.	A.I.M.,	20	Student	2.87	
5.	R.G.B.	A.I.M.,	19	,,	5.75	
6.	T.R.W.	A.I.M.,	20	,,	11.0	
7.	G.E.W.	A.I.M.,	18	,,	2.87	
8.	B.S.	H.F.,	...	,,	8.6	11.5—a week before when she was unwell.
9.	K.S.	H.F.,	...	Student	2.8	
10.	J.S.	H.F.,	...	,,	2.8	
11.	R.S.G.	H.F.,	...	,,	Trace	
12.	L.K.	Ch.F.	...	,,	5.7	
13.	P. Guha	H.F.,	...	,,	2.8	
14.	P. Ghosal	H.F.,	...	,,	1.15	
15.	M.M.	H.F.,	...	,,	2.8	
16.	M.D.	H.F.,	...	,,	2.8	
17.	M.S.	H.F.,	...	,,	2.8	
18.	S.A.W.	M.M.,	21	,,	2.8	
19.	R.R.	H.M.,	20	,,	2.8	
20.	J.R.K.	Parsee M.,	21	,,	5.7	
21.	D.K.S.	H.M.,	18	,,	Trace	
22.	A.R.	H.M.,	19	,,	2.8	
23.	P.S.P.	H.M.,	19	,,	1.15	
24.	B.K.J.	H.M.,	20	,,	5.7	
25.	R.N.S.	H.M.,	21	,,	2.8	
26.	G.R.M.	H.M.,	20	,,	Trace	
27.	P.L.K.	H.M.,	20	,,	2.8	
28.	S.N.	H.M.,	18	,,	1.15	
29.	S.N.G.	H.M.,	19	,,	Trace	
30.	S.S.	H.M.,	18	,,	2.8	
31.	J.B.R.	H.M.,	20	,,	Trace	
32.	H.C.C.	H.M.,	52	Chemist	1.15	
33.	I.C.	M.M.,	25	,,	1.72	
34.	A.S.J.	M.M.,	24	Lab. Asstt.	1.15	
35.	U.C.M.	H.M.,	50	,,	Trace	
36.	P.N.J.	H.M.,	36	Lab. boy	1.15	
37.	J.M.C.	H.M.,	19	,,	1.15	
38.	K.C.P.	H.M.,	20	,,	1.15	
39.	M.H.	M.M.,	18	,,	Trace	
40.	P.C.M.	H.M.,	35	Lab. Asstt.	2.8	

H.M. = Hindu male. M.M. = Muslim male. H.F. = Hindu female. A.I.M. = Anglo-Indian male. Ch.F. = Christian female.

It will be found in the above table that in 26 out of 40 cases (or 65%) the value ranged between 1.15 and 2.87 mg. and in 8 cases it was only 'a trace', or in other words, the value did not exceed 2.87 mg. in 34 cases or 85% of the total number of cases. The high figure 11.0 mg. was found in only one cse, an Anglo-Indian student. In another case (No. 8) a Punjabee girl student, the first examination gave 11.5 mg. when she was slightly indisposed after a touch of 'flue' and on the second examination a week later it was 8.6 mg. when she was quite fit. The mean of these 40 figures is 2.45 mg. per 100 cc. of blood. It is, therefore, evident that the normal value in the case of Indians (2.45 mg.) does not differ very much from that of the Europeans (3.7 mg.). '

The most significant feature of the results obtained so far by determining the normal value is that very low figures were given by those who were absolutely healthy and higher figures by those who were of slightly indifferent health such as after an attack of flue. In diabetic subjects the figures were very high and found to vary from 11.5 to 46.0 mg. per 100 cc. of blood (vide Table II).

<center>TABLE II</center>
<center>*Diabetic subjects not addicted or exposed to alcohol.*</center>
<center>*Figures indicate mg. of alcohol and other dichromate reducing substances*</center>
<center>*(expressed as alcohol) per 100 cc. of fresh blood.*</center>
<center>Mean = 28.12.</center>

Serial No.	Name.	Sex.	Result.	Remarks.
1.	B.M.	H.M.	46.0	
2.	A.G.	H.M.	43.12	
3.	M.A.G.	M.M.	34.50	
4.	S.R.	H.M.	19.25	Not passing sugar in the urine—after treatment.
5.	P.B.	H.M.	11.50	
6.	N.N.G.	H.M.	14.37	

The results in the above table indicate that the interfering substances such as ketone bodies (acetone, aceto-acetic acid, β-hydroxy butyric acid, etc.) and not alcohol are responsible for these high figures. As the condition of health of an unknown person charged for drunkenness may be indifferent or definitely diseased or undergoing treatment with certain medicines such as hexamine, which gives rise to formaldehyde, another interfering substance, the dichromate reducing figure obtained by Southgate's method usually considered as representing the amount of alcohol is certainly fallacious. It is, therefore, not out of the way to suggest that these results point out conclusively that it is essential to adopt the modified method of Koselka and Hine (2) in all cases of suspected drunkenness. It consists in treating the mixed vapours (containing alcohol and the interfering substances) with HgO in the presence of an alkali which eliminates the acetone and other dichromate reducing substances from the alcohol.

While determining the normal alcohol content of the blood of the members of the staff of this department it was observed that those who had been working in the Alcohol Section of this Laboratory had had higher alcohol-content than in those working in the other sections of the department (vide Table III).

TABLE III

Healthy persons not addicted to alcohol but working in the Excise (alcohol) Laboratory and exposed to vapours of alcohol for 4-5 hours at a stretch every day.

Figures indicate mg. of alcohol and other dichromate reducing substances (expressed as alcohol) in 100 cc. of fresh blood.

Serial No.	Name.	Age, Sex etc.		Occupation.	Result.	Remarks.
1.	S.S.G.	H.M.,	44	Chemist	7.4	
2.	A.P.B.	H.M.,	25	,,	5.75	
3.	N.N.C.	H.M.,	38	Lab. Asstt.	6.90	
4.	P.C.M.	H.M.,	35	,,	5.70	Same case as No. 40 in Table I.
5.	G.S.	H.M.,	24	Lab. boy	8.6	
6.	A.K.	M.M.,	32	Chemist	4.6	Same case as No. 2 in Table I.
7.	H.R.	M.M.,	24	Lab. boy	6.9	
8.	A.W.	M.M.,	22	,,	6.9	
9.	J.D.	H.M.,	32	,,	8.6	
10.	E.B.	M.M.,	19	,,	9.2	
11.	M.H.	M.M.,	19	,,	8.6	
12.	B.B.	H.M.,	30	,,	11.5	
13.	S.K.B.	H.M.,	45	Chemist	6.9	

All the thirteen persons shown in the above Table are attached to the Excise (alcohol) section of this laboratory where about 11,000 samples of wines, spirits, tinctures and other alcoholic preparations are analysed annually. There is no exhaust fan nor any other system of artificial ventilation in this section of the laboratory and as such its atmosphere gets almost saturated with vapours of alcohol.

It is interesting to note that all the cases shown in Table III gave high figures, the range being 4.6 to 11.5, in comparison with those shown in Table I. The figures falling within this range of variation were also obtained in six cases given in Table I, where they formed only 15% of the total number of cases, whereas they formed 100% in Table III. The mean of these figures is 7.5 mg. against 2.45 of those in Table I.

The facts that the air of this laboratory gets charged with plenty of alcohol vapour and that alcohol vapour, like ether or chloroform, is readily absorbed through the respiratory tract, are obviously responsible for the uniformly high alcohol content of the blood of those working in this laboratory. The number of cases examined is of course not sufficient for statistical purposes, but the fact that every case, without exception, gives a high figure, raising the average from 2.45 to 7.5 mg. cannot possibly be ignored. The case No. 2 in Table I whose alcohol content of the blood was only a trace when he was working in the Opium Section of the laboratory, but went up to 4.6 (case No. 6 in Table III) when he took up work in the alcohol section, lends particular support to our view. This was further confirmed by an experiment on one of us (K.N.B.) whose blood was examined for its alcohol content before he exposed himself to vapours of alcohol

and again examined for the second time after he worked for 4 hours in the vapours of alcohol in the Excise Laboratory. The first result was only 'a trace' and the second was 3.45 mg. per 100 cc. of blood.

To attain a concentration of 7.5 mg. of alcohol per 100 cc. of blood (as absorbed through the respiratory tract) it will be necessary to ingest about two drachms of brandy or whisky by the mouth [Evans and Jones, (1)], and as 12 mg. of alcohol (per 100 gm. of blood) is oxidised per hour in the blood [Southgate & Carter, (5)] the maintaining of a steady concentration of 7.5 mg. for 3 or 4 hours at a stretch, would involve absorption of quite a large amount of alcohol by inhalation which, if expressed in terms of ounces of a liquor administered by the mouth, would amount to about 2 drachms of brandy or rum per hour. By actual experiments we have also seen that a dose of half an ounce (14 cc.) of brandy given by the mouth raised the alcohol content of the blood of one of us (H.D.G.) from 1.15 to 14.3 mg. in 90 minutes, or in other words, a dose of two drachms of brandy or rum would raise the alcohol content to 6.6 mg. per 100 cc. of blood.

In those who do not take alcohol even as a therapeutic measure on religious or other grounds, its administration by the respiratory route, if it is equally efficacious therapeutically, appears to us to be feasible of being carried out successfully. It may be stated in this connection that the practice of bubbling oxygen through rectified spirit for administration of both oxygen and alcohol to bad cases of pneumonia which was in vogue in the hospitals of Calcutta about 35 years ago but now discarded, appears, on the basis of these findings, to be quite rational although adopted empirically by the physicians of those days.

SUMMARY

1. Estimation of alcohol in the blood gives some idea of the amount of alcohol consumed and the degree of intoxication (drunkenness) produced thereby.

2. The dichromate-reduction method of Southgate is usually adopted for this purpose. The result not only indicates the amount of alcohol but other volatile oxidisable substances present in the blood, as for instance, ketone bodies and certain drugs.

4. Healthy persons working in an atmosphere containing vapours of alcohol imbibe alcohol through the respiratory tract and show a steady concentration of alcohol in the blood, varying from 4.6 to 11.5 mg. per 100 cc. of blood (the average being 7.5 mg.).

5. The maintaining of a steady concentration of 7.5 mg. of alcohol per 100 cc. of blood for 3-4 hours at a stretch is equivalent to about 7 cc. or quarter ounce of brandy or rum administered by the mouth per hour.

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